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**Trifluoracetolysis,
a new method for structural studies on complex carbohydrates**



Lund 1978

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a new method for structural studies on complex carbohydrates

av

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fil.mag., Ld

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TRIFLUORACETOLYSIS, A NEW METHOD FOR STRUCTURAL STUDIES
ON COMPLEX CARBOHYDRATES

by

Bo Nilsson

Lund 1978



TO
ANNE-MARIE

ABSTRACT

A new method is described for the isolation of the carbohydrate chains of glycoproteins by treatment with mixtures of trifluoroacetic anhydride and trifluoroacetic acid. These mixtures may also be used to N-deacetylate 2-acetamido-2-deoxy glycosides, and degrade oligosaccharides containing reducing 2-acetamido-2-deoxy sugars. The trifluoroacetolysis method has been applied to reducing sugars, methyl glycosides, oligosaccharides, polysaccharides, a glycopeptide and the glycoprotein fetuin, the carbohydrate structure of which has been determined.

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This thesis is a summary of the following papers (I-VI):

- I Studies on the stability of reducing sugars towards trifluoroacetylation:
A method for specific elimination of 2-acetamido-2-deoxy-hexose
residues at reducing ends of oligosaccharides
Bo Nilsson and Sigfrid Svensson
Carbohydr. Res., in press
- II Studies on the reactivity of methyl glycosides, oligosaccharides and
polysaccharides towards trifluoroacetylation
Bo Nilsson and Sigfrid Svensson
Submitted for publication
- III A new method for N-deacetylation of 2-acetamido-2-deoxy-sugars
Bo Nilsson and Sigfrid Svensson
Carbohydr. Res., in press
- IV Specific cleavage of the glycosidic bond to L-threonine in 3-O- β -D-
-glucopyranosyl- α -L-fucopyranosyl-L-threonine by trifluoroacetylation
Bo Nilsson and Sigfrid Svensson
Submitted for publication
- V Structural studies on the carbohydrate portion of fetuin
Bo Nilsson, Nils E. Nordén and Sigfrid Svensson
Submitted for publication
- VI A new method for degradation of the protein part of glycoproteins. Iso-
lation of the carbohydrate chains of asialofetuin
Bo Nilsson and Sigfrid Svensson
Submitted for publication

INTRODUCTION

Carbohydrates exhibit a profound diversity in Nature, existing for example as relatively simple oligosaccharides in plants and milk, as complex polysaccharides in starch, glycogen, bacterial and plant cell walls, as sulphated polysaccharides in glycosaminoglycans, as polar components of glycolipids, as constituents of glycopeptides and glycoproteins, as an integral part of the genetic material DNA and RNA, and as a constituent of several enzyme cofactors. Frequently the function of the carbohydrate part of the molecule is unknown, and many proteins have carbohydrate prosthetic groups varying in amounts from less than 1% to 85% of the total molecular weight. The biological functions of glycoproteins in the living organism vary over a wide range of activities, for example glycoproteins found in plasma have functions in transport, enzymology, immunology, blood coagulation, and freezing point depression. Most enzymes are glycoproteins and at the cellular level glycoproteins may be involved in inter- and intracellular transport, cell-cell recognition, and cell structure. But many of the glycoproteins have an unknown function of which the major component of fetal calf serum, fetuin, is an example.

The biological role of the carbohydrate portion of glycoproteins is in most cases unknown, but some functions may be pointed out. The carbohydrate chains are polar groups and may play a role in the conformation and the solubility of the glycoprotein. A major factor in the uncertainty surrounding the biological function of glycoproteins is the limited knowledge available regarding the total structure of the carbohydrate prosthetic groups, which in turn precludes the formulation of mechanisms of biosynthesis, degradation and function.

Earlier structural studies on the carbohydrate chains in glycoproteins were frequently incomplete due to the lack of suitable methods. Great progress has been made over the last few years in the methodology

for isolation of carbohydrates from glycoproteins and in structural analysis of carbohydrates. Structural analysis of the carbohydrate chains in glycoproteins usually starts with attempts to isolate the carbohydrate chains, frequently based upon reaction with base or treatment with proteolytic enzymes. The isolated carbohydrate chains or glycopeptides were then subjected to sugar and methylation analysis in order to determine the components and the linkage types.

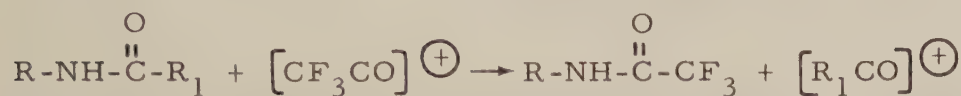
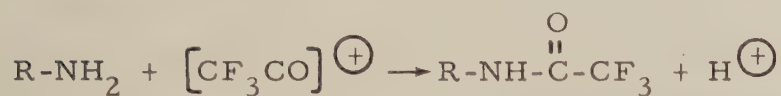
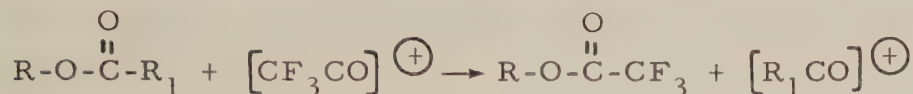
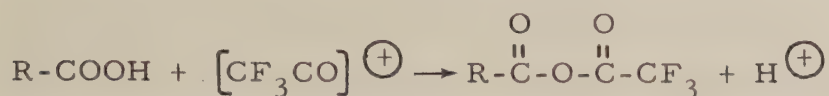
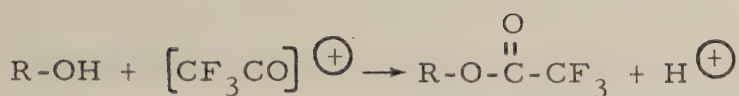
Determination of the sequence is the most difficult part in structural analysis of carbohydrates but several methods are available, such as treatment with glycosidases (1), partial hydrolysis (2), acetolysis (3), Smith degradation (4) and keto degradation (5).

A structural analysis also includes determination of the anomeric configuration which can be accomplished by the use of specific glycosidases (1), chromium trioxide oxidation (6) or spectroscopic methods (7).

The method presented here, trifluoroacetolysis, is based upon introduction of strongly electron-withdrawing groups in the sugar residues to stabilize the glycosidic bonds. The carbohydrate chains in a glycoprotein are cleaved off from the polypeptide chain and the protein is degraded by transamidation. In the first part of this summary the reactivity of model substances towards trifluoroacetolysis is presented. The second part deals with the isolation and structural studies on the two types of carbohydrate chains found in the glycoprotein fetuin.

1. TRIFLUOROACETOLYSIS

Trifluoroacetolysis is performed in a mixture of trifluoroacetic anhydride (TFAA) and trifluoroacetic acid (TFA). This mixture gives rise to the trifluoroacetylum ion $[\text{CF}_3\text{CO}]^+$ which reacts with some common functional groups as follows:



Trifluoroacetic anhydride was first used in carbohydrate chemistry in 1948 (8). It was found that trifluoroacetic anhydride mixed with a carboxylic acid is a powerful acylating mixture. This mixture is suitable for conversion of an alcohol to its carboxylic ester e.g. formation of acetates from acetic acid and trifluoroacetic anhydride (9). The mixture of trifluoroacetic anhydride and acetic acid has also been used in acetolysis of methylene acetals of alditols (10).

O-Trifluoroacetylation is effected by warming the alcohol in trifluoroacetic anhydride in the presence of sodium trifluoroacetate (11). The O-trifluoroacetyl derivatives are stable when dry but hydrolyse rapidly in the presence of water (autocatalytically with liberation of trifluoroacetic acid)(12). O-Trifluoroacetyl groups are also easily removed by 1) dry methanol (11, 13), 2) mild alkaline aqueous methanol (II), 3) sodium borohydride (I), 4) 50% aqueous acetic acid (I), 5) aqueous acetone (14), and 6) aqueous pyridine (15). Trifluoroacetates have also been used as GLC-derivatives of methyl glycosides (16).

N-Trifluoroacetylation can be accomplished by treatment of a primary or a secondary amine with trifluoroacetic anhydride (17). This has been used in the presence of trifluoroacetic acid, for making derivatives of amino acids (18). The N-trifluoroacetyl groups are more stable than O-trifluor-

acetyl groups but the amine can be regenerated by treatment with sodium borohydride or ammonia in aqueous methanol. The difference in stability of the O- and N-trifluoracetyl groups enables complete removal of the O-trifluoracetyl groups whilst leaving the N-trifluoracetyl derivatives untouched.

1.1 Trifluoracetolysis of reducing sugars (I)

When a hexose is treated with a mixture of trifluoroacetic anhydride and trifluoroacetic acid the hydroxyl groups are rapidly O-trifluoroacetylated giving a mixture of pyranoside and furanoside trifluoroacetates (Fig. 1).

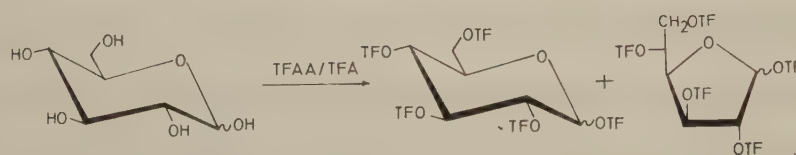


Fig. 1

All hexoses, pentoses and 6-deoxy hexoses were recovered quantitatively after treatment with TFAA/TFA (1:1 and 50:1) at 100° for 48 h and subsequent detrifluoroacetylation, although in TFAA/TFA (1:2) some degradation could be seen (Table I).

The inductive effect of the trifluoroacetyl groups formed presumably stabilizes the sugar and prevents acid catalyzed degradation. Very acid labile sugars such as 2-deoxy-D-erythro-pentose and 2-deoxy-D-arabino-hexose were degraded at all conditions tested (Table I). These results show that an O-trifluoroacetyl group in 2-position is essential for stabilisation of the sugar. It can also be concluded that an O-trifluoroacetyl group in the 6-position has a minimal effect on the stabilisation of the sugar compared to the 2-position.

TABLE I

Sugar	% Recovery ^a		
	1:2 ^b	1:1 ^b	50:1 ^b
<u>D</u> -Glucose	85	100	100
<u>D</u> -Mannose	96	100	100
<u>D</u> -Galactose	82	100	99
<u>D</u> -Xylose	82	99	100
<u>D</u> -Ribose	64	100	99
<u>D</u> -Arabinose	65	100	100
<u>L</u> -Fucose	45	100	100
<u>L</u> -Rhamnose	40	100	100
2-Deoxy- <u>D</u> -arabino-hexose	0	0	0
2-Deoxy- <u>D</u> -erythro-pentose	0	0	0
2-Acetamido-2-deoxy- <u>D</u> -glucose	8	17	85
2-Acetamido-2-deoxy- <u>D</u> -mannose	9	n.d. ^c	90
2-Amino-2-deoxy- <u>D</u> -galactose	12	n.d. ^c	80

^a Determined by GLC-MS after de-O- and de-N-trifluoracetylation by reduction with sodium borodeuteride and subsequent acetylation.

^b Proportions of trifluoroacetic anhydride and trifluoroacetic acid (v/v).

^c n.d. = not determined.

2-Amino- and 2-acetamido-2-deoxy sugars were O-trifluoroacetylated and respectively amidated or transamidated giving per-O- and N-trifluoroacetylated derivatives. These derivatives are largely stable in TFAA/TFA 50:1 but considerably degraded in TFAA/TFA (1:1 and 1:2) (Table I), probably by an acid catalyzed degradation, which is more pronounced as the concentration of acid in the reaction mixture increases.

Reduced sugars and meso-inositol are stable during trifluoroacetylation (TFAA/TFA 1:2, 1:1 and 50:1) as expected.

1.2 Degradation of oligosaccharides containing reducing 2-acetamido-2-deoxy sugars by trifluoroacetytolysis (I)

As mentioned in section 1.1, reducing 2-acetamido-2-deoxy sugars are not stable during trifluoroacetytolysis (TFAA/TFA 1:2, 1:1 and 50:1) in contrast to most other sugars and glycosides (see section 1.3). These facts suggested that a method could be developed for partial degradation of oligosaccharides containing reducing 2-acetamido-2-deoxy sugars. Trifluoroacetytolysis (TFAA/TFA 1:1) of the trisaccharide, α -D-Manp-(1 $\rightarrow$ 3)- β -D-Manp-(1 $\rightarrow$ 4)-D-GlcNAc (19), followed by de-O-trifluoroacetylation by sodium borodeuteride gave the disaccharide alditol, α -D-Manp-(1 $\rightarrow$ 3)-D-Man-OL-1-d, in near quantitative yield (96%) (Fig. 2).

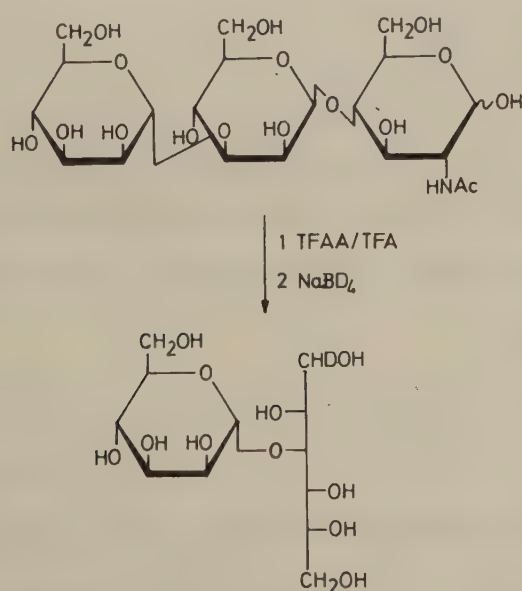


Fig. 2

None of the trisaccharide was found after reduction, acetylation, permethylation and analysis by GLC-MS.

Trifluoroacetytolysis of free 2-acetamido-2-deoxy-D-glucose under the same conditions resulted in a recovery of 17% of the starting material, which may reflect the stabilizing effect of the O-trifluoroacetyl group in the 4-position of the free D-GlcNAc.

Under the same conditions (TFAA/TFA 1:1) oligosaccharides containing reducing 4-O-substituted chitobiose, $\rightarrow 4$)- β -D-GlcNAc $\underline{p}$ -(1 $\rightarrow$ 4)-D-GlcNAc, were degraded in the same way (20). The method has been applied on the glycoprotein fetuin after two consecutive Smith degradations, and the N-glycosidically linked carbohydrate chains were cleaved off from the protein part and degraded as above (see section 2.2).

The O-glycosidically linked carbohydrate chains of fetuin (see section 2.1) are liberated and degraded by trifluoroacetyolysis in TFAA/TFA 1:1 showing that reducing 3-substituted 2-acetamido-2-deoxy sugars are also degraded.

This specific method of degradation is a valuable tool in structural studies on carbohydrates containing reducing 3-O- or 4-O-substituted 2-acetamido-2-deoxy sugars since there are very few methods for degrading oligosaccharides from the reducing end.

Reduced sugars are stable towards trifluoroacetyolysis (see section 1.1) and consequently the trisaccharide alditol α -D-Man $\underline{p}$ -(1 $\rightarrow$ 3)- β -D-Man $\underline{p}$ -(1 $\rightarrow$ 4)-D-GlcNAc-OL was stable under all conditions tested.

1.3 Trifluoroacetyolysis of methyl glycosides (II)

Treatment of a glycoside with a mixture of trifluoroacetic anhydride and trifluoroacetic acid will convert the hydroxyl groups to trifluoroacetates. The glycosidic bond is stabilized due to the strong inductive effect of the O-trifluoroacetyl groups.

All methyl hexopyranosides tested were recovered almost quantitatively after trifluoroacetyolysis (TFAA/TFA 1:1 and 50:1) and subsequent detrifluoroacetylation, since the glycosidic bond is stabilized by O-trifluoroacetyl groups in the 2, 3, 4 and 6-positions (Fig. 3)

The stabilizing effect of a O-trifluoroacetyl group in the 6-position was investigated by trifluoroacetyolysis (TFAA/TFA 1:1 and 50:1) of

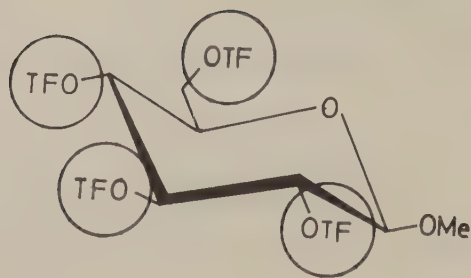


Fig. 3

Me- α -D-xylopyranoside. The recovery of Me- α -D-xylopyranoside was 90% (TFAA/TFA 1:1) and 95% (TFAA/TFA 50:1) which indicated that an O-trifluoracetyl group in the 6-position is not essential for stabilisation of the glycosidic bond. However, Me- α -L-fucopyranoside was recovered only in 65% yield (TFAA/TFA 1:1) and 95% (TFAA/TFA 50:1). The very acid labile Me- β -L-fucofuranoside was recovered in 70% and 97% yield (TFAA/TFA 1:1 and 50:1 respectively). In this case the glycosidic bond is stabilized by O-trifluoracetyl groups in the 2, 3 and 5-positions.

Introduction of strongly electronwithdrawing O-trifluoracetyl groups in the glycone residue will prevent further electrophilic attack by trifluoroacetylum ion on the ring oxygen or the glycosidic oxygen.

The inductive effect of the O-trifluoracetyl groups is strongest adjacent to the anomeric carbon (C-2 in aldoses, Fig. 4), since solvolysis starts with an attack on the C-1 oxygen by the trifluoroacetylum ion (21). However the glycosidic bond is also stabilized by O-trifluoracetyl groups in the aglycone residue, which is discussed in the following section.

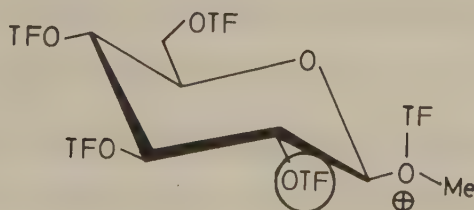


Fig. 4

1.4 Trifluoracetolysis of oligosaccharides and polysaccharides (II)

Disaccharides and linear trisaccharides containing 1→4 and 1→6 hexopyranosidic linkages were stable towards trifluoracetolysis in TFAA/TFA 1:1 and 50:1, since the glycosidic bonds are stabilized by O-trifluoracetyl groups in both the glycone and aglycone moieties. However only 42.8% of the branched trisaccharide, α -D-Galp-(1→3)-[α -L-Fucp-(1→2)]-D-Gal, was recovered after TFAA/TFA 1:1. Free L-Fuc (12.1%) and the disaccharide, α -D-Galp-(1→3)-D-Gal, (40.0%) were also formed. This was expected since Me- α -L-fucopyranoside was partially degraded in TFAA/TFA 1:1 (see section 1.3). In TFAA/TFA 50:1 no degradation occurred and the trisaccharide, α -D-Galp-(1→3)-[α -L-Fucp-(1→2)]-D-Gal, was recovered quantitatively.

The major stabilizing effect of the O-trifluoracetyl groups in the aglycone moiety on the glycosidic bond are shown in Fig. 5 for different types of linkages. O-Trifluoracetyl groups adjacent to the glycosidic oxygen, indicated with circles in the figure, have the dominating stabilizing effect.

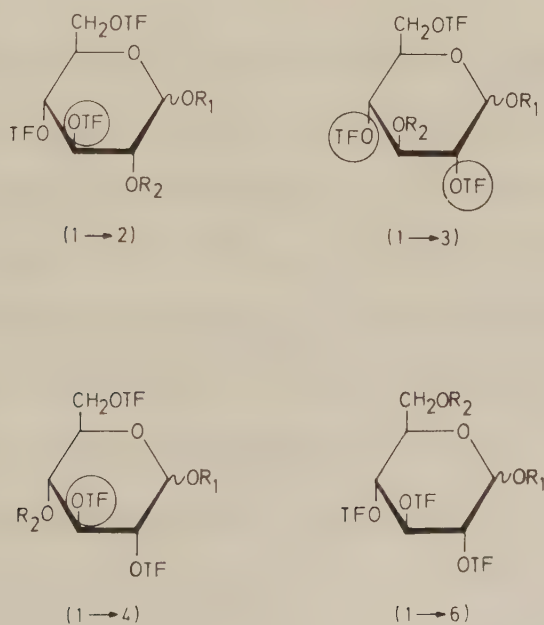


Fig. 5

The polysaccharides dextran, glycogen and guaran were easily dissolved in TFAA/TFA but after 48 h substantial degradation had occurred in TFAA/TFA 1:1. The average molecular weight of dextran had decreased from 158 000 to 25 000. However, when the acid concentration was decreased (TFAA/TFA 50:1) almost no degradation was evident.

1.5 De-N-acetylation of the glycosides of 2-acetamido-2-deoxy sugars by trifluoroacetytolysis (III)

Many glycoproteins contain 2-acetamido-2-deoxy sugars, some of which are directly involved in the sugar-protein linkage. For example 2-acetamido-2-deoxy-D-glucose is N-glycosidically linked to asparagine and 2-acetamido-2-deoxy-D-galactose is O-glycosidically linked to serine or threonine (22). De-N-acetylation is important in structural studies on carbohydrate chains in glycoproteins and other complex carbohydrates containing 2-acetamido-2-deoxy sugars, since the de-N-acetylated product is a starting point for specific degradations such as acid hydrolysis, (2-amino-2-deoxy glycosidic linkages are resistant), periodate oxidation, (oxidation of 4-substituted 2-amino-2-deoxy hexosides) (4) and deamination using nitrous acid (23).

Methods for de-N-acetylation of 2-acetamido-2-deoxy sugars have often been based upon reaction with strong base, examples being saturated aqueous barium hydroxide (24), sodium hydroxide/thiophenolate in aqueous dimethyl sulfoxide (25) and anhydrous hydrazine/hydrazine sulfate (26-28). These methods are not suitable for carbohydrates containing alkali labile sugar residues. Treatment of methyl 2-acetamido-2-deoxy- α -D-glucopyranoside with TFAA/TFA 1:1 or 50:1 initially gives methyl 2-acetamido-2-deoxy-3,4,6-tri-O-trifluoroacetyl- α -D-glucopyranoside [1] (Fig.6). The N-acetyl function is also transamidated [3] probably via the N-trifluoroacetyl-N-acetyl derivative [2] (Fig. 6). This reaction was followed by GLC-MS after de-O-trifluoroacetylation (25% aqueous ammonia in

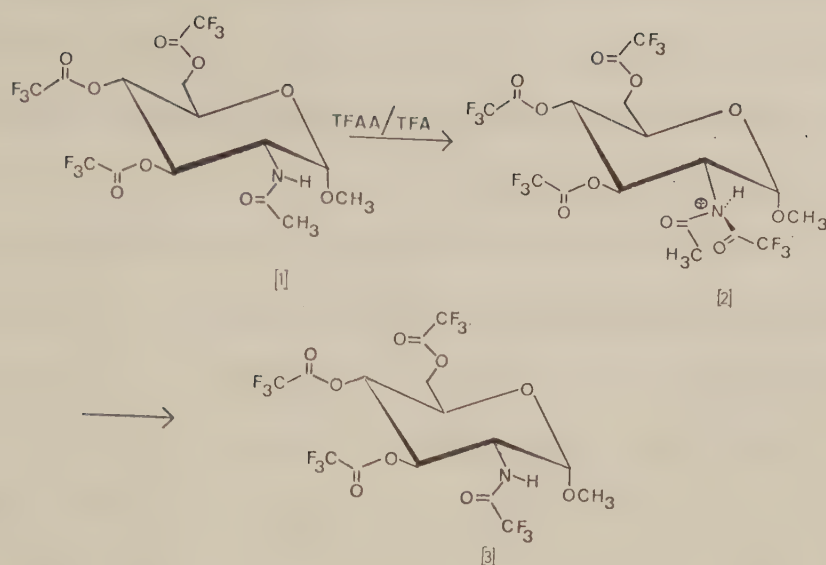


Fig. 6

methanol) and acetylation. After 48 h the N-acetyl function ($\bigcirc$ — $\bigcirc$) was completely converted to an N-trifluoroacetyl function ($\triangle$ — $\triangle$) (Fig. 7).

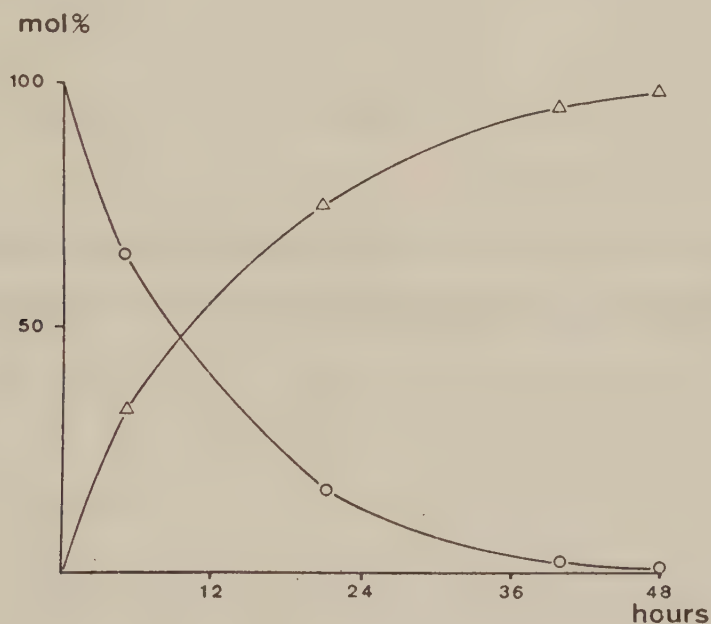


Fig. 7

The mass spectrum of methyl 2-deoxy-2-trifluoroacetamido-3,4,6-tri-O-acetyl- α -D-glucopyranoside (Fig. 8) shows inter alia peaks M-31

(m/e 384), M-60 (m/e 355) and fragments from the molecular ion and from M-31 obtained by consecutive elimination of acetic acid, acetoxyl groups and ketene. The peak at m/e 145 is the triacetoxonium ion always present in peracetylated glycosides (29).

The N-trifluoroacetyl group is easily removed by overnight treatment with sodium borohydride in aqueous ethanol or 1.5 M aqueous ammonia in methanol for 48 h.

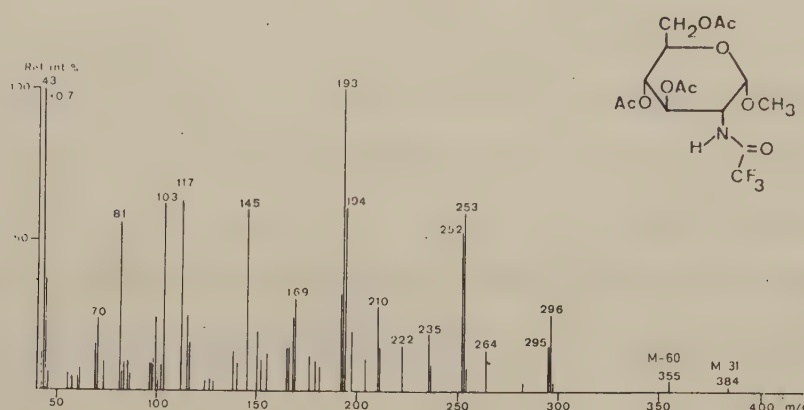


Fig. 8

This new de-N-acetylation method was tested on a capsular polysaccharide from Diplococcus pneumoniae Type 14 (23) having the structure shown in Fig. 9.

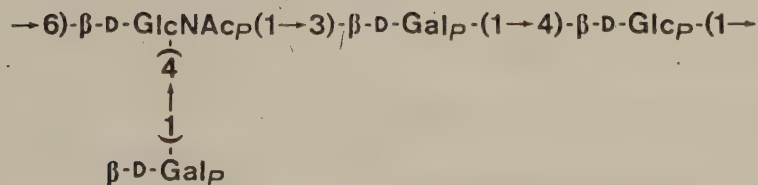


Fig. 9

After trifluoracetolysis (TFAA/TFA 1:1), de-O- and de-N-trifluor-acetylation, deamination, reduction and permethylation, the tetrasaccharide, β -D-Galp-(1→4)- β -D-GlcP-(1→6)-[β -D-Galp-(1→4)]-2,5-anhydro-D-Man-OL, was recovered in more than 90% yield and could be identified by GLC-MS (29).

This method is useful for de-N-acetylation of oligosaccharides and polysaccharides since 1) most polysaccharides are soluble in TFAA/TFA, 2) the reagents are volatile and can be removed by evaporation, 3) the de-N-acetylated product can be purified by gel filtration.

1.6 Trifluoracetolysis of the amino acid glycoside, 3-O- β -D-glucopyranosyl- α -L-fucopyranosyl-L-threonine (IV)

Among the amino acids substituted with carbohydrates, O-glycosidically linked serine and threonine are the most abundant. The usual way of specifically cleaving this type of carbohydrate-protein linkage is by alkaline borohydride treatment (0.8 M NaBH₄ in 0.1 M NaOH at 37° for 72 h) after which reduced sugars are obtained (see section 2) (30).

Trifluoracetolysis (TFAA/TFA 1:1 and 50:1) was tested on β -D-GlcP-(1→3)- α -L-FucP-(1→O)-L-Thr (31) and the fucosyl-threonine linkage was quantitatively cleaved yielding the disaccharide, β -D-GlcP-(1→3)-L-Fuc after de-O-trifluoracetylation by methanol and acetic acid. The mechanism is shown in Fig. 10. After reduction and permethylation the liberated disaccharide was identified and quantitated by GLC-MS (29). The method was also applied on asialofetuin (see section 2.3) and the O-glycosidically-linked carbohydrate chains, β -D-Galp-(1→3)- α -D-GalNAcP-(1→O)-L-Ser (Thr) (see section 2.1) were liberated as discussed earlier (see section 1.2).

The advantage of trifluoracetolysis compared to alkaline borohydride treatment of carbohydrates O-glycosidically linked to serine or threonine is that the oligosaccharides are liberated without being reduced.

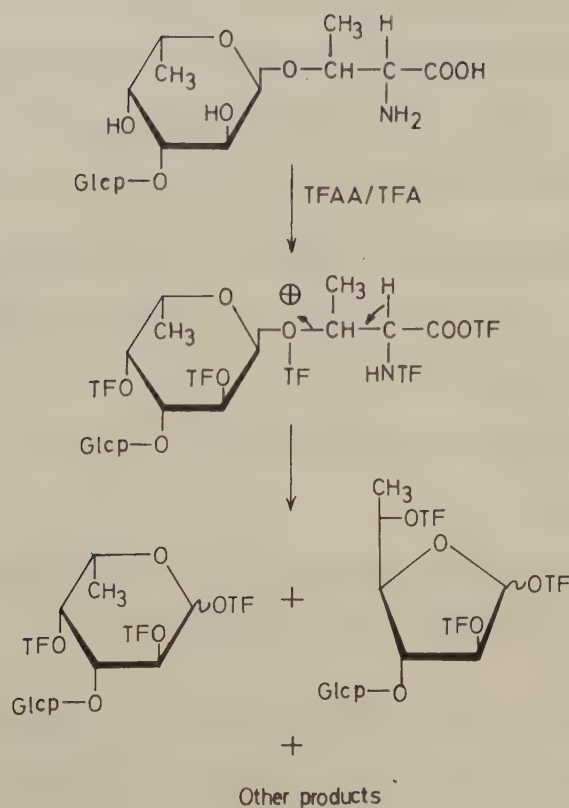


Fig. 10

2. DETERMINATION OF GLYCOPROTEIN STRUCTURE

Glycoproteins are widely distributed in Nature, occurring in all forms of life. Since the carbohydrate content can vary between less than 1% to 85% by weight the isolation procedure cannot be uniform. Common methods for isolation include precipitation by a variety of reagents, ion exchange chromatography and zone electrophoresis (32). Among more than 100 monosaccharides known in Nature, less than 20 have been found in glycoproteins. The commonly occurring sugars are D-galactose, D-mannose, D-glucose, L-fucose, L-rhamnose, D-xylose, L-arabinose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, D-glucuronic acid,

I-iduronic acid and sialic acids, although rarely more than four or five are present in a given glycoprotein. Of about 20 common amino acids, approximately one half are theoretically capable of forming glycopeptides, but only a few have hitherto been found linked to carbohydrates. The three types of carbohydrate-protein linkages characterized so far are N-glycosidic (33, 34), O-glycosidic (31, 35-41), and S-glycosidic (42, 43).

The N-glycosidic type of carbohydrate-protein linkage (Fig. 11) is very common, being found for example in fetuin, transferrin, orosomucoid and ovomucoid. Glycopeptides containing this D-GlcNAc_p-(1→N)-Asn (2-acetamido-1-N-(4-I-aspartyl)-2-deoxy-β-D-glucopyranosylamine) linkage have been obtained from glycoproteins by extensive digestion with proteolytic enzymes (pronase or papain) (44-45). The D-GlcNAc_p-(1→N)-Asn linkage can be hydrolyzed by acid, yielding 1 mole each of aspartic acid, D-glucosamine and NH₃. The glycosylamine bond is stable to mild alkaline treatment, but can be cleaved under strong alkaline conditions (0.2 M NaOH, 100°, 16 h) (47).

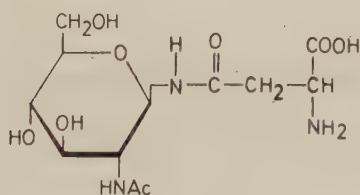


Fig. 11

Another common type of carbohydrate-protein linkage is O-glycosidic, in which N-acetyl-D-galactosamine (35), D-galactose (36), D-mannose (37), I-fucose (31), and D-xylose (38) have been found linked to serine or threonine (exemplified in Fig. 12 by α-D-GalNAc_p-(1→O)-Ser), while D-galactose is the only known sugar O-glycosidically linked to hydroxylysine (Fig. 13) (39).

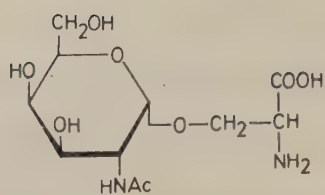


Fig. 12

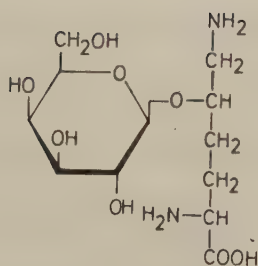


Fig. 13

In the cell wall of plants both D-galactose (40) and L-arabinose (41) (Fig. 14) are found O-glycosidically linked to hydroxyproline .

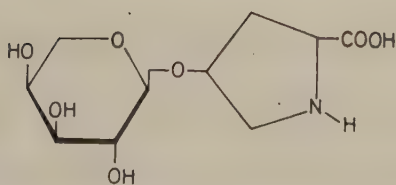


Fig. 14

The O-glycosidic bond to serine or threonine is alkaline labile and reduced oligosaccharides can be isolated from seryl- or threonyl-O-glycosides by alkaline borohydride treatment (30) (Fig. 15) as mentioned in Section 1.6.

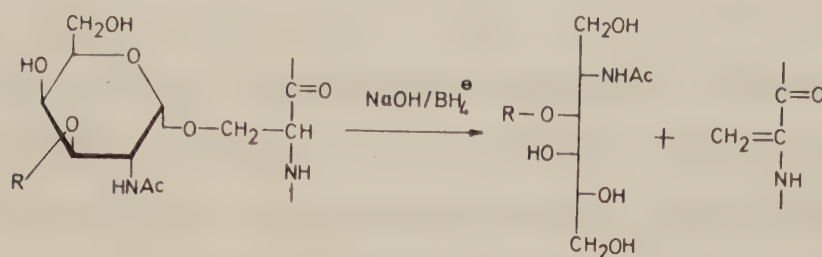


Fig. 15

The S-glycosidic bonds so far reported involve D-galactose (42) or D-glucose (Fig. 16) (43) and cysteine.

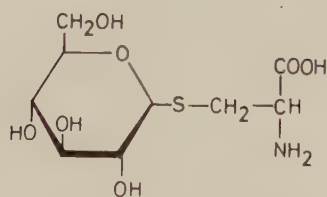


Fig. 16

Fetuin, a glycoprotein of unknown function occurring in large amounts in fetal and newborn calf serum contains both N- and O-glycosidically linked carbohydrate chains and was therefore chosen for study. The usefulness of trifluoracetolysis in the characterization of the carbohydrate chains is outlined in the following sections.

2.1 Structural studies on the O-glycosidically-linked carbohydrate chains of fetuin (V)

Fetuin was first isolated from calf serum by Pedersen in 1944 by adding ammonium sulphate to precipitate the fetuin (48), but later investigations showed that preparations made in this way were not pure (49). A pure and homogeneous preparation can be obtained by low temperature ethanol fractionation (49).

Fetuin has a molecular weight of about 46 000 and contains about 23% carbohydrate as sialic acid, D-galactose, D-mannose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine. Two types of carbohydrate chains exist in the molecule, one O-glycosidically linked to serine and/or threonine and the other N-glycosidically linked to asparagine.

The O-glycosidically linked carbohydrate chains were isolated after alkaline borohydride treatment (30), dialysis, ion exchange and fractionation on a G-25 column (Fig. 17), after which four carbohydrate-containing fractions were obtained.

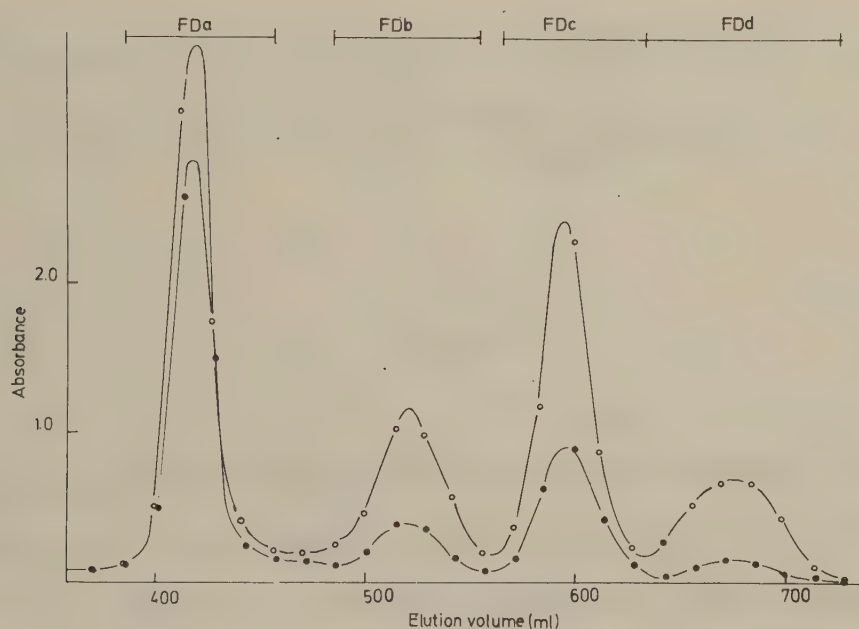


Fig. 17

One of these fractions eluted with the void volume. The retarded fractions contained a tetrasaccharide, α -NANA-(2 $\rightarrow$ 3)- β -D-Galp-(1 $\rightarrow$ 3)-[α -NANA-(2 $\rightarrow$ 6)]-D-GalNAc-OL, a trisaccharide, α -NANA-(2 $\rightarrow$ 3)- β -D-Galp-(1 $\rightarrow$ 3)-D-GalNAc-OL, and sialic acid positive material in the monosaccharide region.

The tetra- and trisaccharide structures were confirmed by sugar (50-52) and methylation analysis (53) of the sialo and asialo compounds. These oligosaccharides were also permethylated and analysed by GLC-MS after desialylation by both acid hydrolysis (54) and treatment with neuraminidase (54). The (1 $\rightarrow$ 3) linkage in the permethylated Galp-(1 $\rightarrow$ 3)-GalNAc-OL is indicated by the presence of fragments m/e 378, m/e 133 and the absence of m/e 174 (Fig. 18).

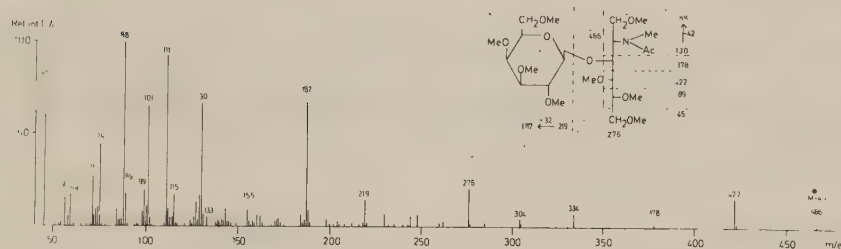


Fig. 18

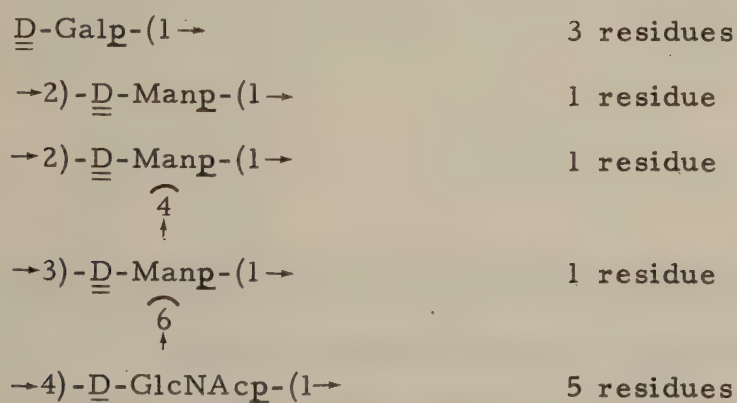
The anomeric configurations in the tri- and tetrasaccharide were determined by neuraminidase treatment and optical rotation.

It is tempting to speculate that the presence of sialic acid positive material together with a disialyltetrasaccharide and structurally similar monosialyltrisaccharide is a result of alkaline "peeling" reaction after the release of the O-glycosidically linked carbohydrate chains.

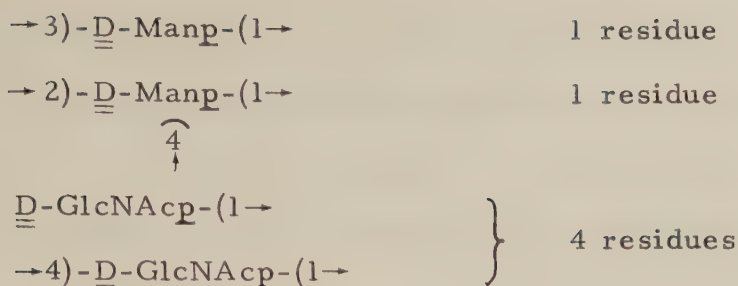
2.2 Structural studies on the N-glycosidically-linked carbohydrate chains of fetuin (V)

The void volume fraction from the G-25 column (section 2.1) and the non-dialysable material (FND) contained the N-glycosidically linked carbohydrate chains still bound to the protein. Sugar and methylation analysis of both fractions gave the same molar composition and the proportion of carbohydrate present in both cases was higher than in the original fetuin, indicating some degradation of the polypeptide chain during the alkaline borohydride treatment. The void volume fraction has a molecular weight less than approximately 10 000 (dialysable). The carbohydrate content increased to about 80% after digestion of the combined void volume peak and FND by papain (FNDP). After desialylation of part of this material (AFNDP) both fractions were then further analysed in parallel by two consecutive Smith degradations (4), trifluoroacetylolysis (55) (see section 2.3), chromium trioxide oxidation (6) and sugar and methylation analysis followed by GLC-MS (50-53).

Sugar analysis showed AFNDP contained D-Gal, D-Man and D-GlcNAc in the ratio 3:3:5 and methylation analysis indicated that these sugars were linked as follows:



After the first Smith degradation the sugar analysis of the larger component was D-Man:D-GlcNAc = 2:4 indicating loss of three D-Gal residues, one D-Man and one D-GlcNAc residue. These remaining sugars were linked as follows, as determined by methylation analysis.



A smaller component was also found and characterized by GLC-MS after reduction and permethylation (Fig. 19), as D-GlcNAcp-(1→2)-glycerol-1-d which accounts for the loss of one D-Man and one D-GlcNAc residue mentioned above (the glycerol comes from degradation of the 2-O-substituted D-Man residue).

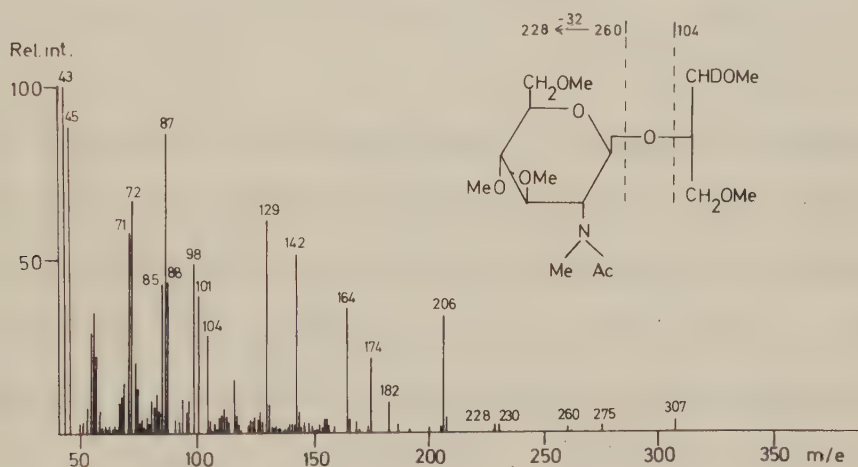
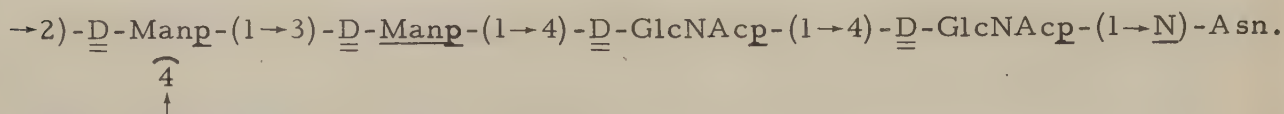


Fig. 19

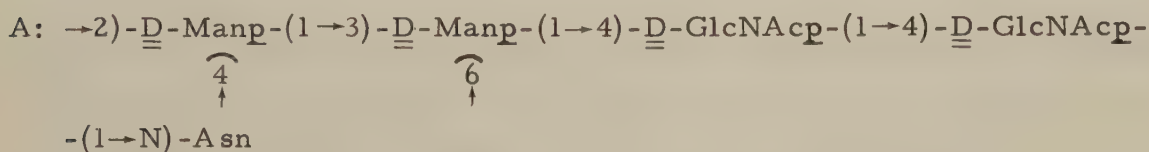
A further Smith degradation of the larger fragment above resulted in the formation of a compound containing D-Man and D-GlcNAc in equal proportions. Trifluoroacetyolysis of this compound (TFAA/TFA 1:1) yielded D-Manp-(1→3)-D-Man. Since TFAA/TFA is known (section 1.2) to cleave the D-GlcNAcp-(1→N)-Asn linkage and degrade reducing D-GlcNAc, it can be concluded that the product after the second Smith degradation was D-Manp-(1→3)-D-Manp-D-GlcNAcp-D-GlcNAcp-(1→N)-Asn. Methylation analysis indicated that internal D-GlcNAc residues were 4-O-sub-

stituted and that the two D-Man residues were 3-O-substituted and 2,4-di-O-substituted. Therefore the following structural elements must be present:



Methylation analysis of AFNDP before Smith degradation indicated the presence of a 3,6-di-O-substituted D-Man residue which can only correspond to the underlined D-Man in the structure above.

Thus the structure of the N-glycosidically linked carbohydrate chains of fetuin must consist of the following elements:



B: $\underline{\underline{D}}-\text{GlcNAcp}-(1\rightarrow 2)$ -glycerol must come from $\underline{\underline{D}}-\text{GlcNAcp}-(1\rightarrow 2)-\underline{\underline{D}}-\text{Manp}-(1\rightarrow$ which, being a product of Smith degradation, must in turn arise from $\underline{\underline{D}}-\text{Galp}-(1\rightarrow 4)-\underline{\underline{D}}-\text{GlcNAcp}-(1\rightarrow 2)-\underline{\underline{D}}-\text{Manp}-(1\rightarrow$

The remaining elements determined by sugar and methylation analysis not accounted for by A and B above are two $\underline{\underline{D}}-\text{Galp}-(1\rightarrow$ residues (C) and two $\rightarrow 4)-\underline{\underline{D}}-\text{GlcNAcp}-(1\rightarrow$ residues (D).

The attachment of B, C and D to A can occur in a variety of ways, but a limitation can be made since only B or one of the $\underline{\underline{D}}-\text{Galp}-(1\rightarrow$ residues (C) can be attached to the 6-position of the internal D-Man in A. If a $\rightarrow 4)-\underline{\underline{D}}-\text{GlcNAcp}-(1\rightarrow$ residue is attached, which in turn must be substituted, it would not be removed by the first Smith degradation with the result that the methylation analysis would show a 3,6-di-O-substituted D-Man residue. This limitation reduces the number of possible composite structures to three, which are shown in Fig. 20.

Sugar analysis and sialic acid determination of FNDP showed NANA, D-Gal, D-Man and D-GlcNAc in ratio 3:3:3:5. Methylation analysis of FNDP and AFNDP indicated that D-Gal is 3- or 6-O-substituted by NANA.

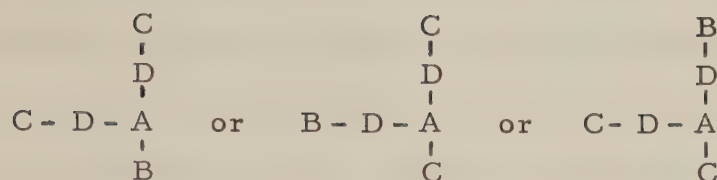


Fig. 20

After the first Smith degradation of FNDP and fractionation, the large component showed on sugar analysis the ratio $\underline{\underline{\text{D}}}\text{-Gal}:\underline{\underline{\text{D}}}\text{-Man}:\underline{\underline{\text{D}}}\text{-GlcNAc} = 1:2:4$ indicating loss of two $\underline{\underline{\text{D}}}\text{-Gal}$ residues, one $\underline{\underline{\text{D}}}\text{-Man}$ residue and one $\underline{\underline{\text{D}}}\text{-GlcNAc}$ residue. Two smaller components were also found and identified by GLC-MS after reduction and permethylation as $\underline{\underline{\text{D}}}\text{-GlcNAcp}-(1\rightarrow2)\text{-glycerol-1-}\underline{\underline{\text{d}}}$ and $\underline{\underline{\text{D}}}\text{-Galp}-(1\rightarrow4)\text{-}\underline{\underline{\text{D}}}\text{-GlcNAcp}-(1\rightarrow2)\text{-glycerol-1-}\underline{\underline{\text{d}}}$ (Fig. 21) in the proportions 1:10. The trisaccharide alditol $\underline{\underline{\text{D}}}\text{-Galp}-(1\rightarrow4)\text{-}\underline{\underline{\text{D}}}\text{-GlcNAcp}-(1\rightarrow2)\text{-glycerol-1-}\underline{\underline{\text{d}}}$ accounts for the loss of one $\underline{\underline{\text{D}}}\text{-Gal}$ residue (3- $\underline{\text{O}}$ -substituted by NANA). The loss of the $\underline{\underline{\text{D}}}\text{-Man}$ and the $\underline{\underline{\text{D}}}\text{-GlcNAc}$ residues have been discussed earlier. After the second Smith degradation sugar analysis gave $\underline{\underline{\text{D}}}\text{-Man}:\underline{\underline{\text{D}}}\text{-GlcNAc} = 2:3$ and after trifluoroacetylation the trisaccharide, $\underline{\underline{\text{D}}}\text{-GlcNAcp}-(1\rightarrow4)\text{-}\underline{\underline{\text{D}}}\text{-Manp}-(1\rightarrow3)\text{-}\underline{\underline{\text{D}}}\text{-Man}$, showing that the product after second Smith degradation was $\underline{\underline{\text{D}}}\text{-GlcNAcp}-(1\rightarrow4)\text{-}\underline{\underline{\text{D}}}\text{-Manp}-(1\rightarrow3)\text{-}\underline{\underline{\text{D}}}\text{-Manp}-(1\rightarrow4)\text{-}\underline{\underline{\text{D}}}\text{-GlcNAcp}-(1\rightarrow4)\text{-}\underline{\underline{\text{D}}}\text{-GlcNAcp}-(1\rightarrow\underline{\underline{\text{N}}})\text{-Asn}$.

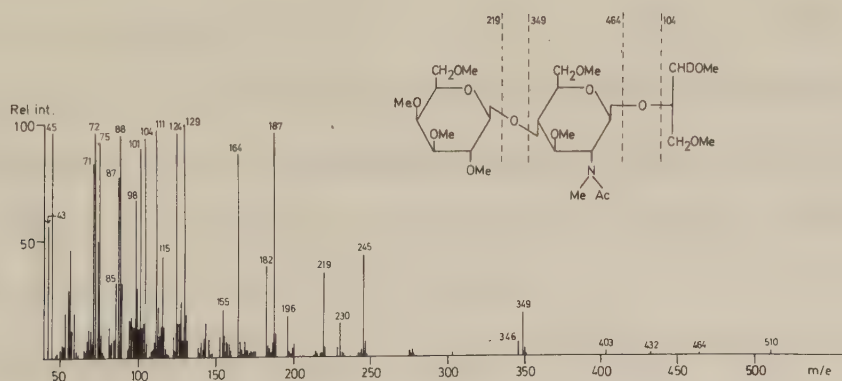


Fig. 21

The data presented above indicates that the following elements are present in the N-glycosidically linked carbohydrate chains of fetuin:

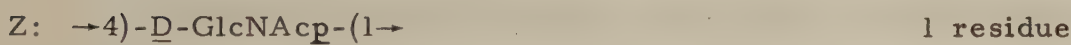
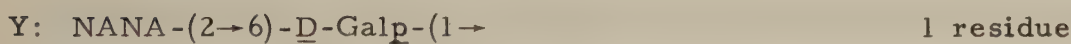
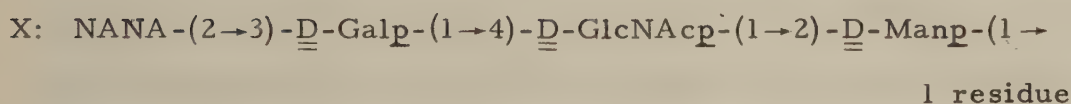
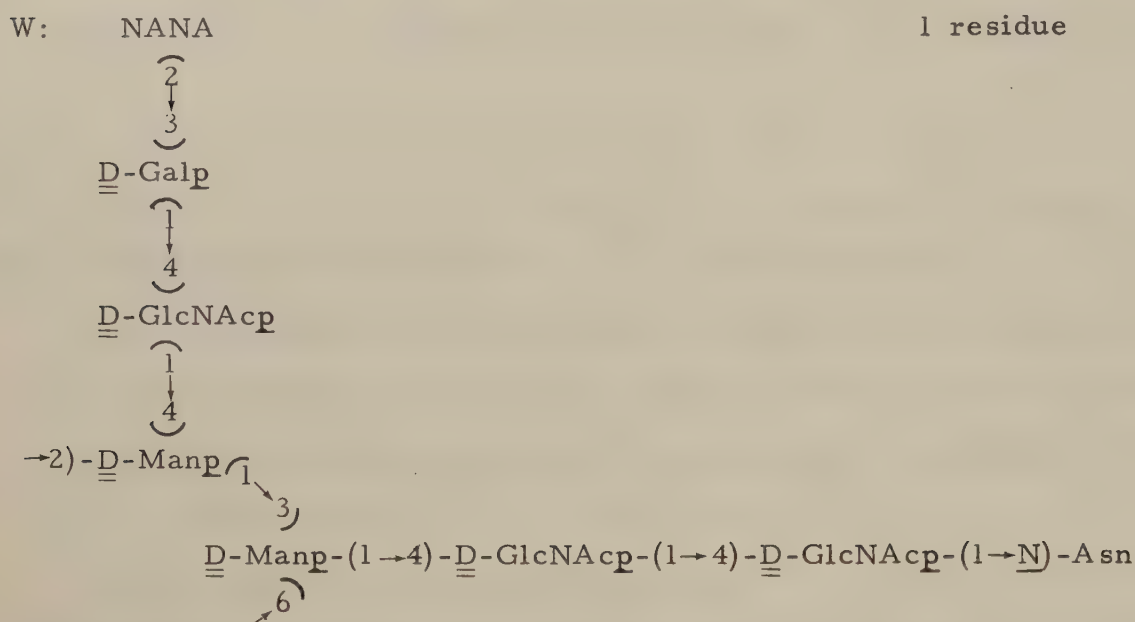


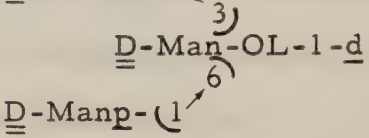
Fig. 22

These four structural elements (W, X, Y and Z) can be combined in several ways, but only two are compatible with the results obtained with FNDP:



Fig. 23

In order to determine the right structure (I or II) and to determine the anomeric configuration, the N-glycosidically linked chains were acetylated and oxidized by chromium trioxide after isolation by trifluoroacetylolysis (section 2.3). Sugar analysis and permethylation of the oxidized material showed a mannose-containing trisaccharide alditol, $\underline{\underline{D}}\text{-Manp-}\underline{\underline{1}}\text{-}$



and fully methylated $\underline{\underline{D}}\text{-GlcNAc-OL}$ when analyzed by GLC-MS. This result shows that the only possible structure is I (Fig. 23).

The N-glycosidically linked carbohydrate chains of fetuin must then have the structure

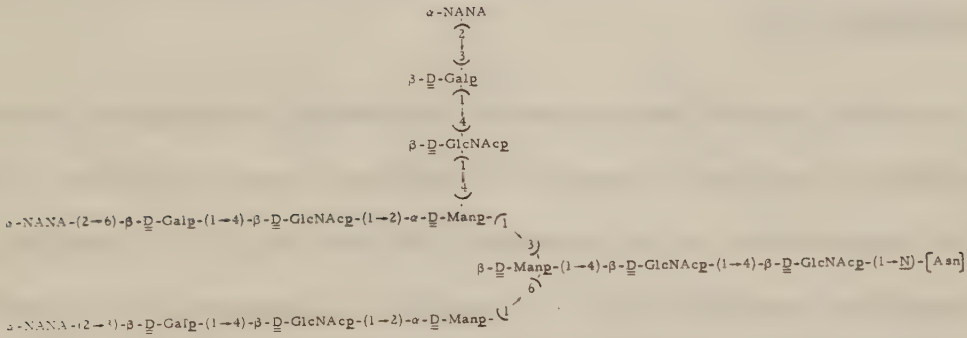


Fig. 24

From the carbohydrate content and the molecular weight it can be estimated that three N-glycosidically linked carbohydrate chains reside in the molecule. After Smith degradation and reduction of AFNDP the disaccharide alditol $\underline{\underline{D}}\text{-GlcNAcp-(1}\rightarrow\text{2)-glycerol-1-d}$ was liberated. However, this disaccharide alditol was also found together with the trisaccharide alditol $\underline{\underline{D}}\text{-Galp-(1}\rightarrow\text{4)-}\underline{\underline{D}}\text{-GlcNAcp-(1}\rightarrow\text{2)-glycerol-1-d}$ after Smith degradation of FNDP. The presence of the disaccharide alditol after Smith degradation of FNDP can be explained by partial hydrolysis of the (2→3)-linked NANA or that a minor part of this NANA residue is (2→6)-linked instead

of (2→3)-linked. The latter explanation is supported by the methylation analysis of FNDP, ^{where} the proportions (2→3)-linked NANA to (2→6)-linked are 1.8:1.1. This indicates some heterogeneity among the N-glycosidically linked carbohydrate chains, caused by the sialic acid substitution, but the majority of the N-glycosidically linked carbohydrate chains have the structure shown in Fig. 24.

The biological function of the frequently complex carbohydrate structures attached to protein molecules is largely unknown. The structure obtained above adds to the limited information presently available in this field and exemplifies the methods now available for use in this complex field of study.

2.3 Isolation of the carbohydrate chains from asialofetuin by trifluoroacetolysis (VI)

In order to perform structural studies on the carbohydrate chains of glycoproteins it is often necessary to remove as much protein as possible due to its interference in the carbohydrate analysis. By repeated digestion with proteolytic enzymes (56) it is possible to remove most of the protein leaving a few amino acids linked to the carbohydrate chain.

Among the chemical methods available for isolation of carbohydrates from glycoproteins, hydrazinolysis (26) is the most common. When this method was applied to fetuin, the N-glycosidically linked carbohydrate chains were isolated free from protein, but some modification of the reducing end was apparent since no reducing GlcNAc was formed. The O-glycosidically linked carbohydrate chains were completely destroyed. Another chemical method for isolation of carbohydrate chains is based upon treatment with NaOH/NaBH₄ (47), but under these conditions the O-glycosidically linked carbohydrate chains of fetuin were destroyed and the N-glycosidically linked chains were liberated in about 75%

yield. Trifluoroacetylation (TFAA/TFA 50:1) of glycoproteins results in cleavage of the peptide bonds by transamidation and stabilisation of the glycosidic bonds in the carbohydrate chains by trifluoroacetylation.

After trifluoroacetylation of asialofetuin in TFAA/TFA 50:1 and subsequent de-O-trifluoroacetylation by methanol and aqueous acetic acid the material was fractionated on a G-25 column and pooled according to the anthrone content (●—●) (Fig. 25).

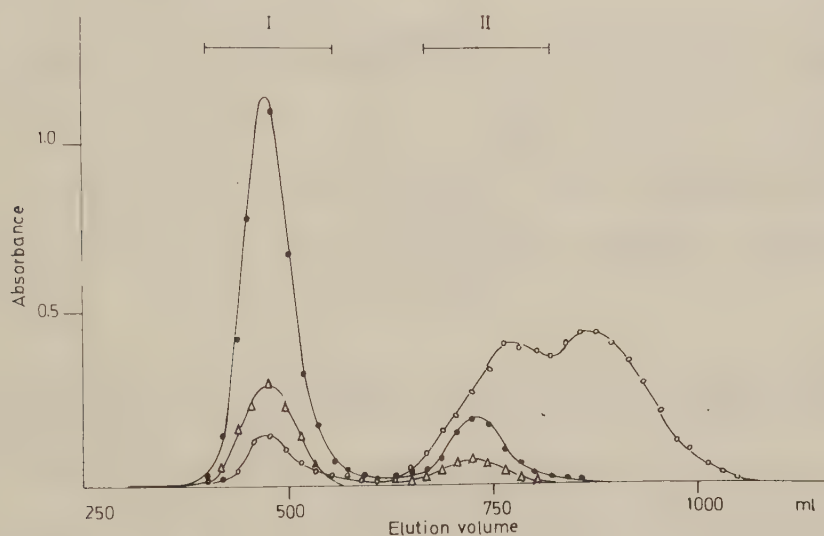


Fig. 25

Fractions I and II were then reconstituted (de-N-trifluoroacetylated by NaBD_4 and N-acetylated) and fractionated on a G-50 (Fig. 26) and

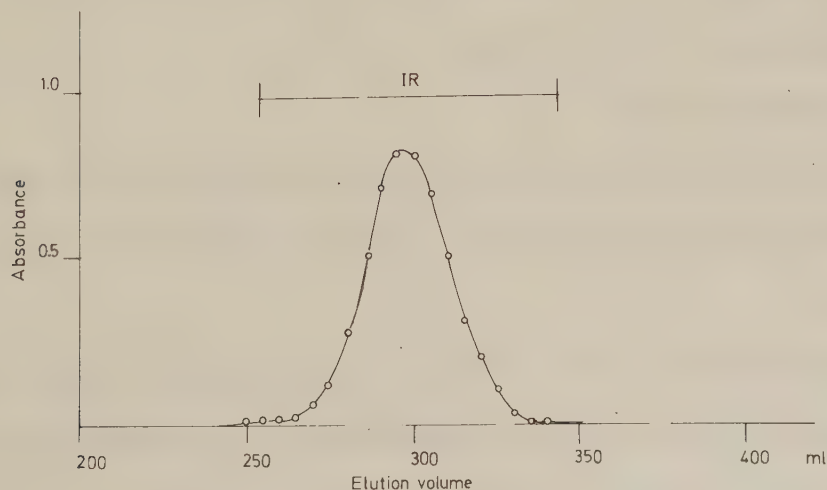


Fig. 26

a G-25 column (Fig. 27) yielding fractions IR and IIR respectively.

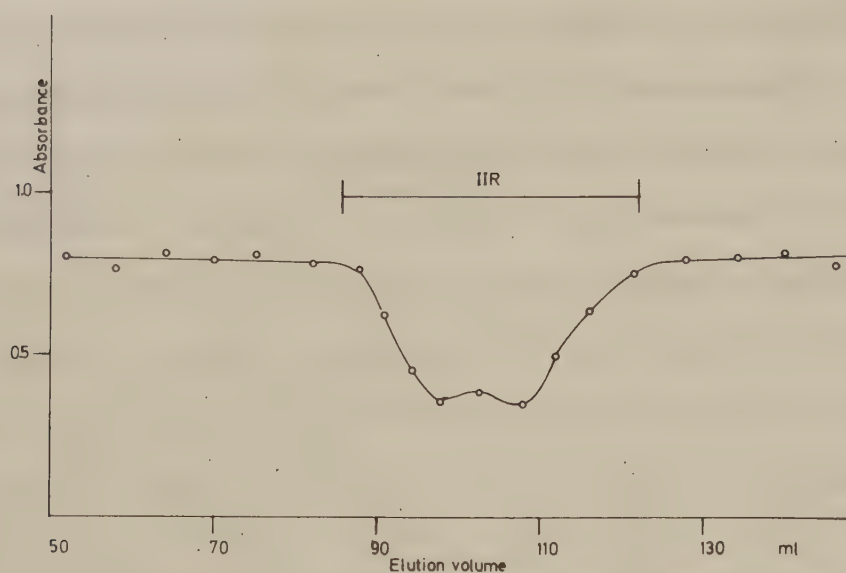


Fig. 27

Sugar and methylation analysis of fraction IR (the N-glycosidically linked chains) indicated partial degradation at the reducing end. The N-glycosidically linked carbohydrate chains (Fig. 24) are cleaved off from the asparagine residue generating a glycosylamine which is then degraded (57), and a reducing D-GlcNAc is formed, which is further degraded as discussed in section 1.2. According to the results obtained in the sugar and methylation analysis of fraction IR three species were found:

- A: $R-\underline{\underline{D}}\text{-Manp}-(1\rightarrow4)-\underline{\underline{D}}\text{-GlcNAcp}-(1\rightarrow4)-\underline{\underline{D}}\text{-GlcNAc-OL-1-d}$
- B: $R-\underline{\underline{D}}\text{-Manp}-(1\rightarrow4)-\underline{\underline{D}}\text{-GlcNAc-OL-1-d}$
- C: $R-\underline{\underline{D}}\text{-Man-OL-1-d}$

R represents the rest of the N-glycosidically linked carbohydrate chain. Calculation based upon sugar and methylation analysis gave the ratio A:B:C = 2:4:4.

Fraction IIR (Fig. 27) contained the O-glycosidically linked carbohydrate chains, which are probably released by a mechanism similar to the one shown in Fig. 10. The liberated disaccharide, $\beta\text{-}\underline{\underline{D}}\text{-Galp}-(1\rightarrow3)-\underline{\underline{D}}\text{-GalNAc}$

is then partially degraded, as previously discussed, yielding free D-Gal and the disaccharide β -D-Galp-(1→3)-D-GalNAc.

In addition to loss of terminal D-GlcNAc, trifluoroacetylation (TFAA/TFA 1:1) of asialofetuin showed partial degradation at the branch points (55). But TFAA/TFA 1:1 was successfully used after two consecutive Smith degradations of FNDP and AFNDP (section 2.2). The D-GlcNAc α -Asn linkage was cleaved with subsequent degradation of the reducing D-GlcNAc formed (section 1.2).

In conclusion, trifluoroacetylation can at present be used:

- 1) For isolation of carbohydrates from glycoconjugates. Glycosidic bonds are stabilised by O-trifluoroacetyl groups, while proteins are degraded by transamidation. O-Glycosidically and N-glycosidically linked carbohydrates can be isolated.
- 2) In structural studies on carbohydrates. Specific degradation of reducing 2-acetamido-2-deoxy sugars.
- 3) For de-N-acetylation of 2-acetamido-2-deoxy glycosides.

The work described here covers the initial experimentation and development of a new method. Analysis of the reactions with more model compounds and with a greater variety of carbohydrate-containing compounds of biological interest will clarify the mechanisms involved and enable the scope of the method to be fully elucidated.

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STUDIES ON THE STABILITY OF REDUCING SUGARS TOWARDS TRI- FLUORACETOLYSIS: A METHOD FOR SPECIFIC ELIMINATION OF 2-ACETAMIDO-2-DEOXYHEXOSE RESIDUES AT REDUCING ENDS OF OLIGOSACCHARIDES

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In studies of the scope of trifluoracetolysis in structural carbohydrate chemistry, we have developed a new method for N-deacetylation of 2-acetamido-2-deoxy sugar derivatives¹, and also a new technique for the isolation of N- and O-glycosidically linked carbohydrate chains in glycoproteins² based upon the fact that most glycosidic bonds are stable³ during trifluoracetolysis and that proteins are degraded by transamidation. We now report on the stability of reducing sugars towards trifluoracetolysis.

Treatment of pentoses, hexoses, or 6-deoxyhexoses with trifluoroacetic anhydride (TFAA)/trifluoroacetic acid (TFA) in the proportions 1:1 and 50:1, at 100° for 48 h gave the corresponding pertrifluoroacetates in quantitative yields (Table I). When the proportions were 1:2 degradation occurred which was more pronounced for the pentoses and the 6-deoxyhexoses, and was probably induced by acid catalysed elimination of trifluoroacetic acid from the initially formed pertrifluoroacetates. 2-Deoxy-D-erythro-pentose and 2-deoxy-D-arabino-hexose were rapidly destroyed under all of the above conditions. 2-Amino-2-deoxy-D-galactose was presumably first converted into its per-O- and N-trifluoroacetylated deri-

TABLE I Recovery of sugars after trifluoroacetylation under different conditions at 100° for 48 h

Sugar	Recovery (%) ^a		
	1:2 ^b	1:1 ^b	50:1 ^b
<u>D</u> -Glucose	85	100	100
<u>D</u> -Mannose	96	100	100
<u>D</u> -Galactose	82	100	99
<u>D</u> -Xylose	82	99	100
<u>D</u> -Ribose	64	100	99
<u>D</u> -Arabinose	65	100	100
<u>L</u> -Fucose	45	100	100
<u>L</u> -Rhamnose	40	100	100
2-Deoxy- <u>D</u> -arabino-hexose	0	0	0
2-Deoxy- <u>D</u> -erythro-pentose	0	0	0
2-Acetamido-2-deoxy- <u>D</u> -glucose ^d	8	17	85
2-Acetamido-2-deoxy- <u>D</u> -mannose ^d	9	n.d. ^c	90
2-Amino-2-deoxy- <u>D</u> -galactose ^d	12	n.d.	80

^a Determined by g.l.c.-m.s. after de-O- and de-N-trifluoroacetylation by reduction with sodium borodeuteride and reacetylation.

^b Proportions of trifluoroacetic anhydride and trifluoroacetic acid (v/v).

^c Not determined.

^d When analyzed after de-O-trifluoroacetylation with 50% aqueous acetic acid at room temperature for 1 h, followed by acetylation, only per-acetylated 2-deoxy-2-trifluoroacetamidohexoses were found on g.l.c.-m.s.

vative which was largely stable in 50:1 TFAA/TFA but was degraded when the proportions were 1:1 and 1:2. 2-Acetamido-2-deoxy sugars were converted into their pertrifluoroacetates and then gradually transamidated to give the N-trifluoroacetates which were stable in 50:1 TFAA/TFA but severely degraded by the 1:1 and 1:2 reagents. Thus, the presence of a 2-O-trifluoroacetyl group in the pertrifluoroacetates of reducing sugars is essential for stabilisation towards acid catalysed degradation.

The finding that 2-acetamido-2-deoxy sugars were degraded by TFAA/TFA under conditions where hexoses, pentoses, and most glycosides were stable, prompted an investigation of the behaviour of oligosaccharides with a 2-acetamido-2-deoxy sugar at the reducing end. Thus, α -D-Manp-(1→3)- β -D-Manp-(1→4)-D-GlcNAc⁴ gave pertrifluoracetylated α -D-Manp-(1→3)-D-Man in near quantitative yields after trifluoracetolysis with the 1:2 and 1:1 reagents but in only 27% yield with the 50:1 reagent which gave mainly the per-O- and N-trifluoracetylated trisaccharide derivative. Thus, the 4-O-substituted 2-acetamido-2-deoxy-D-glucose residue in the trisaccharide is more labile towards trifluoracetolysis than is 2-acetamido-2-deoxy-D-glucose. This finding is expected since the stabilizing effect of the 4-O-trifluoracetyl group is lost. The specific elimination of reducing 4-O-substituted 2-acetamido-2-deoxy-D-glucose residues from oligosaccharides should be of value in structural carbohydrate chemistry. Preliminary results also suggest that reducing 3-O-substituted 2-acetamido-2-deoxy sugars are similarly eliminated whereas the behaviour of 6-O-substituted reducing 2-acetamido-2-deoxy sugars is more complex.

EXPERIMENTAL

Concentrations were performed under reduced pressure at 40° (bath). G.l.c. was performed on a Perkin-Elmer 3920 instrument equipped with a flame ionization detector and (a) glass columns (2 m x 0.25 cm) packed with 3% ECNSS-M on Chromosorb Q (for alditol acetates at 200°) and (b) SE-30 W.C.O.T. glass capillary columns (25 m x 0.25 mm) (for 2-acetamido-2-deoxy- and 2-deoxy-2-trifluoracetamido-hexitol derivatives at 220° and for permethylated oligosaccharide alditols at 250°). For g.l.c.-m.s. a Varian MAT 311A instrument was used fitted with the appropriate column. Mass spectra were recorded at 70 eV with an ionization current of 3 mA, and an ion source temperature of 150°. The mass

spectra were processed on an on-line computer system (Spectrosystem 100, Varian MAT).

Trifluoroacetytolysis of sugars. - The sugar (4 mg, accurately weighed) and myoinositol (4 mg, as inert internal standard) were treated with TFAA/TFA (5 ml, 1:2, 1:1 or 50:1) at 100° for 48 h in a sealed glass tube. Each cooled mixture was concentrated to dryness, and a solution of the residue in methanol (5 ml) was concentrated to dryness. A solution of the product in 50% aqueous acetic acid was concentrated to dryness. The residue was then dissolved in water (5 ml) and treated with sodium borohydride (25 mg) to effect simultaneous de-N-trifluoroacetylation and reduction. The resulting alditols were treated with acetic anhydride and pyridine to give alditol acetates which were analyzed by g.l.c.-m.s. (Table I).

Degradation of α -D-Manp-(1→3)- β -D-Manp-(1→4)-D-GlcNAc (1). - Mixtures of 1 (2.5 mg) and panose (2.5 mg, as stable internal standard) were treated with TFAA/TFA (5 ml, 1:2, 1:1, 7:1, 20:1, 40:1, and 50:1) at 100° for 48 h in sealed glass tubes. Each reaction mixture was cooled and concentrated. The resulting mixture of trifluoroacetates was treated with methanol and 50% aqueous acetic acid and concentrated to dryness as described above. After reduction with sodium borodeuteride and acetylation the product was permethylated and analyzed by g.l.c.-m.s. (Table II). The identity of permethylated α -D-Manp-(1→3)- β -D-Manp-(1→4)-D-GlcNAc-OL-1-d was demonstrated by comparison of retention time and mass spectrum with those of an authentic sample⁵. The identity of permethylated α -D-Manp-(1→3)-D-Man-OL-1-d was indicated by its mass spectrum which was typical⁶ for a hexp-(1→3)-hex-OL-1-d.

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The authors thank Ingemar Robertsson for excellent technical

TABLE II Trifluoroacetylolysis^a of α -D-Manp-(1→3)- β -D-Manp-(1→4)-D-GlcNAc (1)

TFAA/TFA ^b	1 ^c	α - <u>D</u> -Manp-(1→3)- <u>D</u> -Man ^c
1:2	0	92
1:1	0	96
7:1	17	77
20:1	23	67
40:1	59	37
50:1	67	27

^a At 100° for 48 h.

^b Relative proportions (v/v).

^c Mol % obtained after trifluoroacetylolysis, de-O-, de-N-trifluoroacetylation, and re-N-acetylation; analyzed as their permethylated alditol derivatives.

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STUDIES ON THE REACTIVITY OF METHYL GLYCOSIDES, OLIGO-SACCHARIDES AND POLYSACCHARIDES TOWARDS TRIFLUORACETOLYSIS

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ABSTRACT

Methyl glycosides, oligosaccharides and polysaccharides have been treated with trifluoroacetic anhydride and trifluoroacetic acid under conditions that will effect transamidation. It was found that most glycosides are stable and are converted into pertrifluoroacetates. The reason and prerequisite for stability of glycosides and oligosaccharides during trifluoroacetolysis is discussed.

INTRODUCTION

In studies of methods for the isolation of the carbohydrate chains of glycoproteins we have investigated the possibility of stabilizing the glycosidic bonds in order to cleave the peptide bonds under acid conditions. In the present communication we demonstrate how glycosidic bonds are stabilized under trifluoroacetolysis conditions that will cleave amide linkages¹ (peptide bonds).

RESULTS

Methyl glycosides, oligosaccharides and polysaccharides have been

treated with mixtures of trifluoroacetic anhydride (TFAA) and trifluoroacetic acid (TFA) at 100° for 48 h. The proportions of TFAA and TFA were varied from 1:1 to 50:1 (v/v), under which conditions methyl 2-acetamido-2-deoxy- α -D-glucopyranoside is quantitatively converted into methyl 2-deoxy-2-trifluoroacetamido-3, 4, 6-tri-O-trifluoroacetyl- α -D-glucopyranoside¹. Each compound was subsequently de-O-trifluoroacetylated, characterized and quantitated by g.l.c. -m.s. using the appropriate derivatives.

The methyl glycosides tested were all stable in TFAA/TFA, 50:1, being converted into their corresponding pertrifluoroacetates, and could be quantitatively recovered after de-O-trifluoroacetylation (Table I). In TFAA/TFA 1:1 the more acid labile 6-deoxyhexosides and pentoside were partially hydrolyzed (Table I).

The oligosaccharides were treated with TFAA/TFA and analyzed by g.l.c. -m.s. as their permethylated derivatives after de-O-trifluoroacetylation and reduction. The results are shown in Table II.

TABLE I

Treatment of glycosides with TFAA/TFA at 100° for 48 h

Compound	Recovery (%) ^a	
	TFAA/TFA 1:1	TFAA/TFA 50:1
Me- α - <u>D</u> -Glc _p	> 98	> 98
Me- β - <u>D</u> -Glc _p	> 98	> 98
Me- α - <u>D</u> -Man _p	> 98	> 98
Me- α - <u>D</u> -Gal _p	> 98	> 98
Me- β - <u>D</u> -Gal _p	> 98	> 98
Me- α - <u>D</u> -Xyl _p	90	95
Me- α - <u>L</u> -Fuc _p	65	95
Me- β - <u>L</u> -Fuc _f	70	97

^a The recovery was determined by g.l.c. after de-O-trifluoroacetylation, reduction and acetylation.

TABLE II

Treatment of oligosaccharides with TFAA/TFA at 100° for 48 h

Compound	Recovery (%) ^a	
	TFAA/TFA 1:1	TFAA/TFA 50:1
β -D-Glcp-(1→4)-D-Glc	>98	>98
α -D-Glcp-(1→6)-D-Glc	>98	>98
β -D-Glcp-(1→6)-D-Glc	>98	>98
β -D-Galp-(1→4)-D-Glc	>98	>98
α -D-Glcp-(1→4)- α -D-Glcp-(1→4)-D-Glc	>98	>98
α -D-Glcp-(1→6)- α -D-Glcp-(1→4)-D-Glc	>98	>98
α -D-Galp-(1→3)-[α -L-Fucp-(1→2)]-D-Gal	43	98

^a The recovery was determined by g.l.c.-m.s. after de-O-trifluoroacetylation, reduction and permethylation.

On treatment with TFAA/TFA the polysaccharides (dextran, guaran and glycogen) dissolved and were converted into their trifluoroacetate derivatives. The trifluoroacetylated polysaccharides were detrifluoroacetylated with aqueous, methanolic ammonia and recovered from the reaction mixture by gel chromatography on Sephadex G-25. The polysaccharides were then analyzed by sugar and methylation analysis. Since some depolymerisation of the polysaccharides could be expected, dextran (Mw. 158 000) was trifluoroacetylated in mixtures of TFAA/TFA varying from 1:1 to 50:1, de-O-trifluoroacetylated and subjected to gel chromatography on Sephadex G-200. The results are shown in Fig. 1.

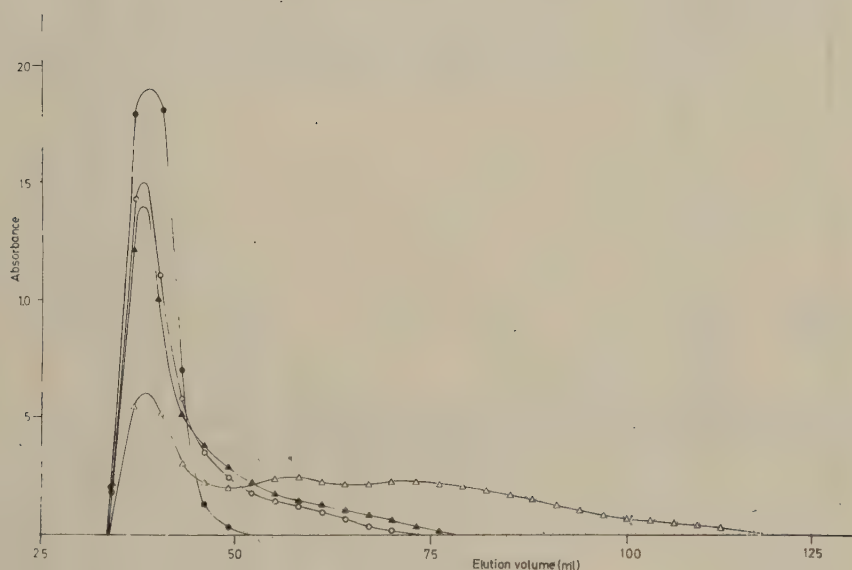


Fig. 1. Gel chromatography on a Sephadex G-200 column (1.5 x 55 cm) of dextran (Mw. 158 000) trifluoroacetylated under various conditions eluted with water. Flow rate, 5 ml/h.

●—● original dextran, $\triangle$ — $\triangle$ treated with TFAA/TFA 1:1, $\blacktriangle$ — $\blacktriangle$ treated with TFAA/TFA 20:1, $\circ$ — $\circ$ treated with TFAA/TFA 50:1. Fractions were assayed with the anthrone reagent⁷.

DISCUSSION

The reagent TFAA/TFA should contain, besides TFAA and TFA, the ions $[\text{CF}_3\text{CO}]^+$ and CF_3COO^- . The reagent is a potent acylating agent under the conditions used and all hydroxyl groups are rapidly converted into O-trifluoracetyl groups. The O-trifluoracetyl functions are strongly electron attracting and thus render the glycosidic acetal function electron deficient. The O-trifluoracetyl groups close to the glycosidic bond should be particularly efficient.

The methyl hexopyranosides were all stable towards trifluoracetolysis and could be quantitatively recovered after de-O-trifluoracetylation. No anomerisation was evident. This finding shows that the O-trifluoracetyl functions in the 2-, 3-, 4-, and 6-positions render the glycosidic bond stable and that further stabilization from the aglycone thus is not needed. The pentopyranoside (Me- α -Xylp) and the 6-deoxy-hexopyranoside (Me- α -Fucp) were stable in TFAA/TFA 50:1, demonstrating that an O-trifluoracetyl group is not needed in the 6-position, even though these glycosides are more sensitive towards acid catalyzed hydrolysis than the hexopyranosides. From these results it is not surprising that the very acid labile furanoside Me- β -Fucf also is stable in TFAA/TFA 50:1 since it has a 2- and a 5-O-trifluoracetyl group adjacent to the acetal oxygens of the glycosidic bond. The acid labile Me- α -Fucp, Me- β -Fucf and Me- α -Xylp were, however, partially solvolized in TFAA/TFA 1:1.

All linear oligosaccharides tested were stable, as expected (Table II) since the glycosidic bond(s) are also stabilized by O-trifluoracetyl groups in the aglycone part of the molecule. The branched trisaccharide α -D-Galp-(1 $\rightarrow$ 3)- $[\alpha$ -L-Fucp-(1 $\rightarrow$ 2)]-D-Gal was recovered in near quantitative yield after trifluoracetolysis using TFAA/TFA in the proportions 50:1 but as expected it could only be recovered in about 40% yield after trifluoracetolysis with TFAA/TFA in the proportions 1:1 due to solvolysis of the

L-fucopyranosidic linkage and formation of α -D-Galp-(1→3)-D-Gal (40%) and L-Fuc (12%).

The polysaccharides dextran, guaran and glycogen were all stable in TFAA/TFA and could easily be recovered, after de-O-trifluoracetylation, in the void volume of a Sephadex G-25 column. Sugar and methylation analyses of the recovered polysaccharide were identical to those of the original polysaccharides. When gel chromatography on Sephadex G-200 was used to detect degradation of trifluoracetylated and de-O-trifluoracetylated dextran it was found that substantial degradation occurred when the trifluoracetolysis was carried out in TFAA/TFA 1:1 and that this degradation was diminished by lowering the proportion of TFA to be virtually absent when trifluoracetolysis was performed in TFAA/TFA 20:1 (Fig. 1).

The stability of glycosides, oligosaccharides and polysaccharides in TFAA/TFA under conditions which transamidate amide (peptide) bonds makes it possible to use the reagent for isolation of carbohydrate chains from glycoproteins². Since most oligosaccharides and polysaccharides are brought into solution by the reagent it should be possible to use trifluoracetolysis to extract carbohydrates from biological material. Other compounds, such as proteins and fats are broken down by the reagent and can easily be removed. Studies to evaluate the usefulness of the reagent TFAA/TFA in the isolation of carbohydrate structures are in progress.

EXPERIMENTAL

General methods. - Concentrations were performed under diminished pressure at bath temperatures not exceeding 40°C. G.l.c. was performed on a Perkin-Elmer 3920 instrument fitted with flame ionization detectors. Separations were performed on (a) glass columns (2 m x 0.5 cm) containing 3% ECNSS-M on Gas Chrom Q (80/100 mesh) at 200°C (alditol

acetates and peracetylated glycosides), (b) glass capillary columns (25 m x 0.25 mm) wall-coated with SE-30 (LKB, Stockholm, Sweden) at 180-330°C (permethylated oligosaccharide alditols). For g.l.c.-m.s. the columns above were used in a Varian MAT 311A combined g.l.c.-m.s. instrument. Glass capillary columns were connected directly to the ion source of the instrument. The mass spectra were recorded at an ionization potential of 70 eV, an ionization current of 3 mA, and an ion source temperature of 120°C. All data were processed by an on-line computer system (Spectro-system 100, Varian MAT).

Treatment of methyl glycosides with TFAA/TFA. - The methyl glycoside (5 mg) and myo-inositol (as internal standard) were treated with TFAA/TFA (1:1 or 50:1, v/v) (2 ml) at 100° in a serum flask (10 ml) sealed with a rubber cap. After 48 h the reaction mixture was cooled and evaporated to dryness. The residue was dissolved in 1 M ammonia in aqueous methanol (2 ml) and evaporated to dryness. The residue was reduced and acetylated with acetic anhydride/pyridine and analyzed by g.l.c.-m.s.^{3, 4} (Table I).

Treatment of oligosaccharides with TFAA/TFA. - The oligosaccharides (5 ml) and maltose (as inert internal standard) were treated as above with TFAA/TFA. After evaporation of the reaction mixture it was reduced with sodium borohydride (10 mg) in 90% aqueous ethanol (5 ml). After processing as above, the residue was permethylated and analyzed by g.l.c.-m.s.⁵ (Table II).

Treatment of polysaccharides with TFAA/TFA. - The polysaccharide (10 mg) was treated with TFAA/TFA (1:1, v/v) (5 ml) as described above. After de-O-trifluoracetylation with ammonia in aqueous methanol the residue was purified by gel chromatography on Sephadex G-25 (2 x 45 cm) (eluted with water).

All polysaccharides were eluted with the void volume, free of reagents. After lyophilization the polysaccharides were analyzed by

g.l.c.-m.s. after sugar^{3, 4} and methylation⁶ analysis. Dextran (10 mg) was also treated with TFAA/TFA (20:1 and 50:1, v/v) and subjected to gel chromatography on Sephadex G-200 (1.5 x 55 cm) after de-O-trifluor-acetylation (Fig. 1).

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Abbreviations

Me- α - $\underline{\underline{D}}$ -Glc_p, methyl- α - $\underline{\underline{D}}$ -glucopyranoside; $\underline{\underline{D}}$ -Gal, $\underline{\underline{D}}$ -galactose; $\underline{\underline{D}}$ -Xyl, $\underline{\underline{D}}$ -xylose; $\underline{\underline{L}}$ -Fuc, $\underline{\underline{L}}$ -fucose; $\underline{\underline{D}}$ -Man, $\underline{\underline{D}}$ -mannose; $\underline{\underline{D}}$ -Glc, $\underline{\underline{D}}$ -glucose.

A NEW METHOD FOR N-DEACETYLATION OF 2-ACETAMIDO-2-DEOXY Sugars

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N-Deacetylation of 2-acetamido-2-deoxysugars can be accomplished using strong base in aqueous solution¹ or in methyl sulphoxide² under drastic conditions. Hydrazinolysis with anhydrous hydrazine or anhydrous hydrazine together with hydrazine sulphate³⁻⁵ are sometimes useful methods.

We now report a new N-deacetylation procedure based upon transamidation with trifluoroacetic anhydride/trifluoroacetic acid followed by cleavage of the resulting O- and N-trifluoroacetyl functions.

Treatment of methyl 2-acetamido-2-deoxy- α -D-glucopyranoside with 1:1 trifluoroacetic anhydride-trifluoroacetic acid (TFAA/TFA reagent) at room temperature rapidly gave methyl 2-acetamido-2-deoxy-3,4,6-tri-O-trifluoroacetyl- α -D-glucopyranoside but at 100° for 48 h transamidation occurred to give methyl 2-deoxy-2-trifluoroacetamido-3,4,6-tri-O-trifluoroacetyl- α -D-glucopyranoside, probably via the N-trifluoroacetyl-N-acetyl derivative. The reaction was monitored by g.l.c.-m.s. after de-O-trifluoroacetylation and acetylation (Fig. 1). After 48 h, no starting material remained and methyl 3,4,6-tri-O-acetyl-2-trifluoroacetamido-2-deoxy- α -D-glucopyranoside was obtained in near quantitative yield.

Methyl 2-deoxy-2-trifluoroacetamido- α -D-glucopyranoside could be readily and quantitatively de-N-trifluoroacetylated with methanolic ammonia

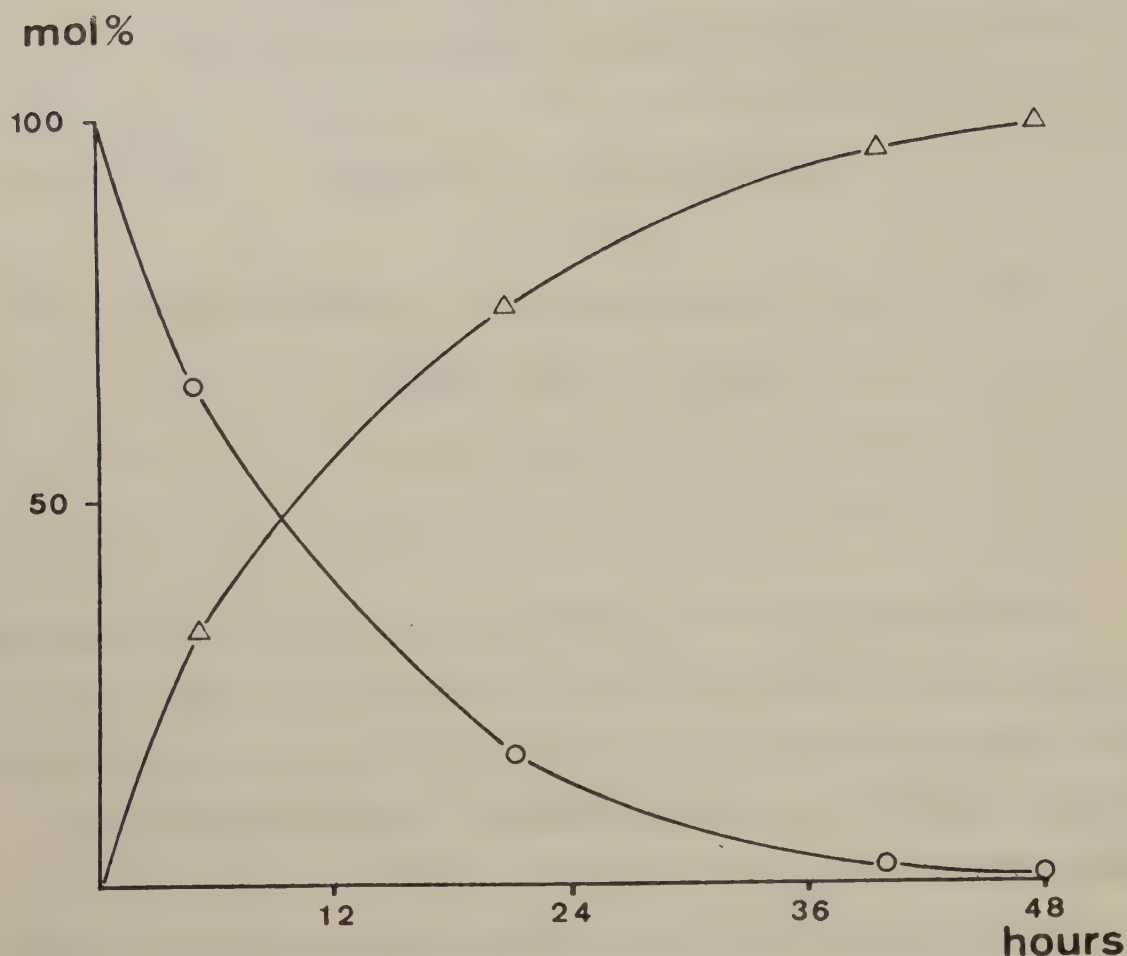
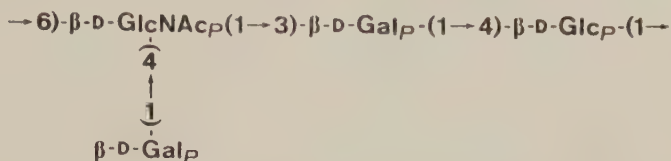


Fig. 1. Treatment of methyl 2-acetamido-2-deoxy- α -D-glucopyranoside with TFAA/TFA at 100°C. Analysis of the reaction mixture after de-O-trifluoroacetylation and reacetylation. Methyl 2-acetamido-2-deoxy- α -D-glucopyranoside (○—○) and methyl 2-deoxy-2-trifluoroacetamido- α -D-glucopyranoside (△—△).

or ethanolic sodium borohydride.

N-Deacetylation is a prerequisite for nitrous acid degradation of complex carbohydrates containing 2-acetamido-2-deoxysugars and since the glycosidic linkages of hexopyranosides, pentopyranosides, 6-deoxyhexopyranosides, and 6-deoxyhexofuranosides are stable⁶ in the TFAA/TFA mixture the new N-deacetylation method was applied to a model polysaccharide.

The capsular polysaccharide⁷ from Diplococcus pneumoniae Type 14, which has the structure



was treated with the TFAA/TFA reagent and subsequently with aqueous sodium borohydride. Nitrous acid degradation⁷ of the de-N-acetylated polysaccharide gave a product which contained D-glucose and D-galactose in the ratio 1:1.9 and only a trace of 2-acetamido-2-deoxy-D-glucose, demonstrating that de-N-acetylation was >95%. After reacetylation, sugar analysis and methylation analysis showed that no significant degradation of the polysaccharide had occurred during the de-N-acetylation. If some degradation of the polysaccharide does occur, a 40:1 TFAA/TFA reagent should be used which will effect transamidation at 100° for 48 h with a minimum of degradation.

The new method should complement other de-N-acetylation procedures, and will be especially useful when the carbohydrate structure is sensitive to strong base.

EXPERIMENTAL

Gel chromatography was performed on columns (3 x 140 cm and 2 x 45 cm) of Sephadex G-25 (fine) by elution with water. Optical rotations were measured using a Perkin-Elmer polarimeter Model 241. For g.l.c. a Perkin-Elmer 3920 instrument fitted with a column of 3% OV-1 on Chromosorb W (80-100 mesh) at 180° was used. G.l.c.-m.s. was performed on a Varian MAT 311A instrument fitted with a glass capillary column (25 m x 0.25 mm) wall-coated with SE-30. Mass spectra were recorded at 70 eV with an ionization current of 3mA and an ion source temperature of 150°.

Methyl 2-deoxy-2-trifluoroacetamido- α -D-glucopyranoside (1). -

Methyl 2-acetamido-2-deoxy- α -D-glucopyranoside (0.8 g) was treated with TFAA/TFA reagent (1:1, 10 ml) at 100° for 48 h in a sealed tube. The resulting dark solution was cooled, concentrated to dryness, and a solution of the residue in methanol was basified with aqueous 25% ammonia. After 1 h at room temperature the solution was concentrated to dryness and the residue was extracted with water (5 ml). The extract was eluted from a column (3 x 140 cm) of Sephadex G-25 (fine) with monitoring by polarimetry. Methyl 2-deoxy-2-trifluoroacetamido- α -D-glucopyranoside (807 mg, 82%), which was eluted in front of three coloured bands, when crystallized from acetone-light petroleum (b.p. 40-60°), had m.p. 136-138°, $[\alpha]_D^{20} +99^\circ$ (c 1, water). (Found: C, 36.5; H, 5.1; N, 4.7; F, 19.1; $C_9H_{14}NF_3O_6$ calc.: C, 37.4; H, 4.9; N, 4.8; F, 19.7%). Ir (KBr) showed a single absorption in the carbonyl region at 1710 cm^{-1} (NHCOCF₃).

The mass spectrum of the triacetate of 1 contained inter alia peaks for (M-OMe)⁺ at m/e 384.0845 (calc. for $C_{14}H_{17}F_3NO_3$, 384.0851) and for (M-AcOH)⁺ at m/e 355.0843 (calc. for $C_{13}H_{16}F_3NO_7$, 355.0847).

De-N-trifluoroacetylation of 1. - (a) With ammonia in aqueous methanol. A solution of 1 (940 μ g) and perseitol (930 μ g, internal standard) in 1.5 M ammonia in methanol:water (4:1) was stored at room temperature for 48 h. No starting material then remained and 2-amino-2-deoxy- α -D-glucopyranoside had been formed in a quantitative yield as determined by g.l.c.-m.s. of the acetylated reaction product.

(b) With sodium borohydride in aqueous ethanol. To a solution of 1 (940 μ g) and perseitol (930 μ g, internal standard) in 90% aqueous ethanol (2 ml) was added sodium borohydride (15 mg). After 10 h at room temperature only trace amounts of starting material remained and 2-amino-2-deoxy- α -D-glucopyranoside had been formed in a near quantitative yield as determined by g.l.c.-m.s. of the acetylated reaction product.

N-Deacetylation of the capsular polysaccharide S-14⁷. - The capsular polysaccharide (50 mg) was heated with TFAA/TFA reagent (1:1, 10 ml) at 100° for 48 h in a sealed tube. The resulting dark, clear solution was cooled, concentrated, and a solution of the residue in 1.5 M ammonia in methanol-water (4:1, 10 ml) was stored for 1 h then concentrated to dryness. The product was dissolved in water (10 ml) and sodium borohydride (50 mg) was added. After 10 h at room temperature the solution was acidified with glacial acetic acid and boric acid was removed by codistillation with methanol. The N-deacetylated polysaccharide (40 mg) was isolated by chromatography on a column (2 x 45 cm) of Sephadex G-25 (fine).

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SPECIFIC CLEAVAGE OF THE GLYCOSIDIC BOND TO $\underline{\underline{L}}$ -THREONINE IN
 $3\text{-}\underline{\underline{O}}\text{-}\beta\text{-}\underline{\underline{D}}\text{-GLUCOPYRANOSYL-}\alpha\text{-}\underline{\underline{L}}\text{-FUCOPYRANOSYL-}\underline{\underline{L}}\text{-THREONINE}$ BY
 TRIFLUORACETOLYSIS

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We have recently shown that most glycosidic bonds are stable¹ under trifluoracetolysis conditions that will effect transamidation². In the present communication we wish to report specific cleavage of the glycosidic bond to $\underline{\underline{L}}$ -threonine in $3\text{-}\underline{\underline{O}}\text{-}\beta\text{-}\underline{\underline{D}}\text{-glucopyranosyl-}\alpha\text{-}\underline{\underline{L}}\text{-fucopyranosyl-}\underline{\underline{L}}\text{-threonine}$ $\left[\beta\text{-}\underline{\underline{D}}\text{-Glc}\underline{\underline{p}}\text{-(1}\rightarrow\text{3)-}\alpha\text{-}\underline{\underline{L}}\text{-Fuc}\underline{\underline{p}}\text{-(1}\rightarrow\text{O)-}\underline{\underline{L}}\text{-Thr}\right]^3$ as a model for the release of $\underline{\underline{O}}$ -glycosidically linked carbohydrate chains in glycoproteins.

$\beta\text{-}\underline{\underline{D}}\text{-Glc}\underline{\underline{p}}\text{-(1}\rightarrow\text{3)-}\alpha\text{-}\underline{\underline{L}}\text{-Fuc}\underline{\underline{p}}\text{-(1}\rightarrow\text{O)-}\underline{\underline{L}}\text{-Thr}$ together with panose (as internal standard) was treated with trifluoroacetic anhydride/trifluoroacetic acid (TFAA/TFA), 1:1 and 50:1, at 100°C for 48 h. These conditions have been shown earlier to effect transamidation². The resulting product was de- $\underline{\underline{O}}$ -trifluoroacetylated by treatment with methanol and acetic acid and analyzed by g.l.c.-m.s. after reduction with sodium borohydride and permethylation. Only one component (beside the internal standard) was obtained with the mass spectrum shown in Fig. 1.

From the mass spectrum, using the A- and J-series of fragmentation and the fragments obtained by α -cleavage of the permethylated alditol chain⁴, the compound is permethylated $\text{hexp-(1}\rightarrow\text{3)-6-deoxy-hexitol}$ (Fig. 1). Since there is no reason to expect anomerisation¹ the compound must be $\beta\text{-}\underline{\underline{D}}\text{-Glc}\underline{\underline{p}}\text{-(1}\rightarrow\text{3)-}\underline{\underline{L}}\text{-Fuc-OL}$. This compound was obtained in

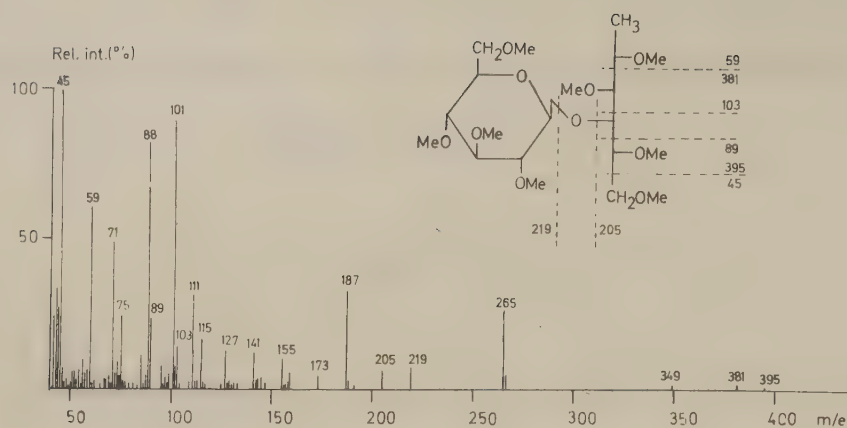


Fig. 1. Mass spectrum of the component obtained after trifluoroacetylation of β -D-Glcp-(1 $\rightarrow$ 3)- α -L-Fucp-(1 $\rightarrow$ O)-L-Thr, detrifluoroacetylation, reduction (NaBH_4) and permethylation. The A- and J-series of fragmentation as well as the α -cleavage fragments of the alditol part are shown in the figure.

theoretical yield (within the experimental error) at both trifluoroacetylation conditions tested. These results show that the glycosidic bond to the L-threonine residue is cleaved during trifluoroacetylation presumably via an acid catalyzed elimination reaction (Fig. 2).

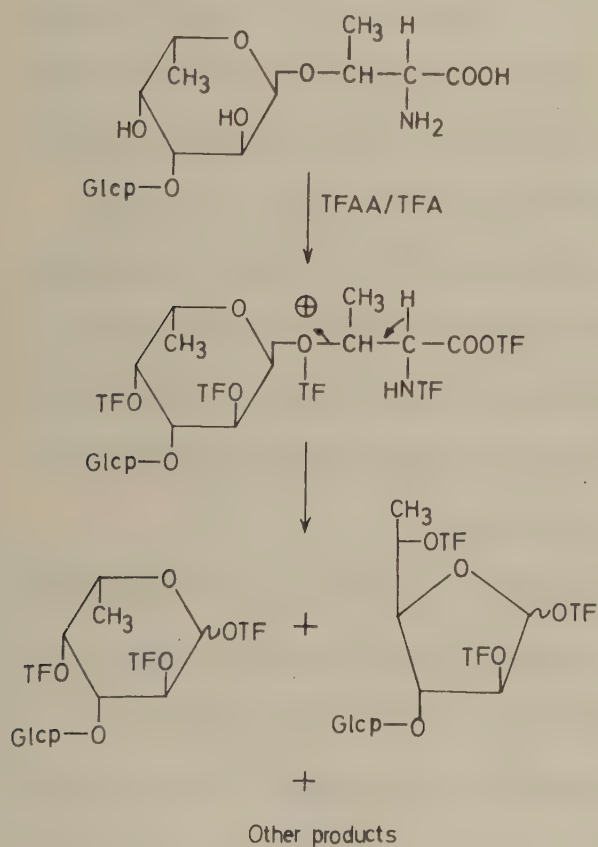


Fig. 2. Suggested mechanism for acid catalyzed cleavage of the glycosidic linkage to L-threonine in β -D-Glcp-(1 $\rightarrow$ 3)- α -L-Fucp-(1 $\rightarrow$ O)-L-Thr.

When trifluoracetolysis was used to release the O-glycosidically linked carbohydrate chains from asialofetuin which have the structure β -D-Galp-(1→3)- α -D-GalNAcp-(1→O)-Ser or Thr^{5, 6}, the condition TFAA/TFA 1:1 yielded mainly pertrifluoracetylated D-Gal⁷ which is expected since a reducing hexNAc residue is degraded under these conditions⁸. When the proportion TFAA/TFA 50:1 was used instead to release the O-glycosidically linked carbohydrate chains from asialofetuin a mixture of pertrifluoracetylated D-Gal and pertrifluoracetylated β -D-Galp-(1→3)-D-GalNHCOCF₃ in the proportions 1:10 was obtained.⁹

The present model study shows that trifluoracetolysis should be of interest for the isolation of O-glycosidically (to serine or threonine) linked carbohydrate chains from glycoproteins and glycopeptides.

EXPERIMENTAL

Concentrations were performed under reduced pressure at bath temperatures not exceeding 40°C. G.l.c. was performed on a Perkin-Elmer 3920 instrument fitted with a flame ionization detector. Separations were carried out on a glass capillary column (25 m x 0.25 mm) wall-coated with SE-30 (LKB Products, Sweden) at 200°-320°. For g.l.c.-m.s. the column above was used in a Varian MAT 311A combined g.l.c.-m.s. instrument. The glass capillary column was connected directly to the ion source of the instrument. The mass spectra were recorded at an ionization potential of 70 eV, an ionization current of 3 mA and an ion source temperature of 120°C. All data were processed by an on-line computer system (Spectrosystem 100, Varian MAT).

Trifluoracetolysis of β -D-Glcp-(1→3)- α -L-Fucp-(1→O)-L-Thr. - To about 1 mg of β -D-Glcp-(1→3)- α -L-Fucp-(1→O)-L-Thr (accurately weighed) and panose (as internal standard) was added TFAA/TFA (1:1 or 50:1, v/v, 2 ml) and the reaction mixture was heated at 100° for 48 h in

a sealed glass tube (Caution: Corrosive mixture under pressure.). After cooling the reaction mixture was concentrated to dryness. The residue was dissolved in methanol (5 ml) and evaporated to dryness. The residue was dissolved in 50% aqueous acetic acid (5 ml) and after 1 h at room temperature the solution was evaporated to dryness. The resulting product was reduced (NaBH_4), permethylated¹⁰ and analyzed by g.l.c.-m.s.⁴

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STRUCTURAL STUDIES ON THE CARBOHYDRATE PORTION OF FETUIN

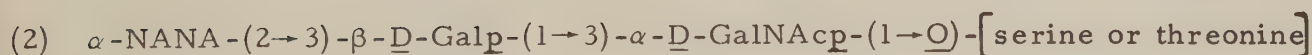
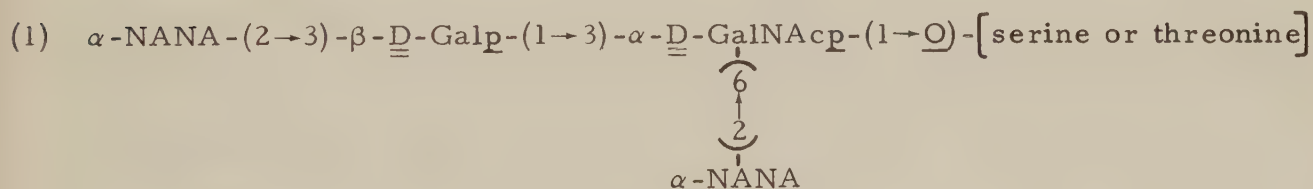
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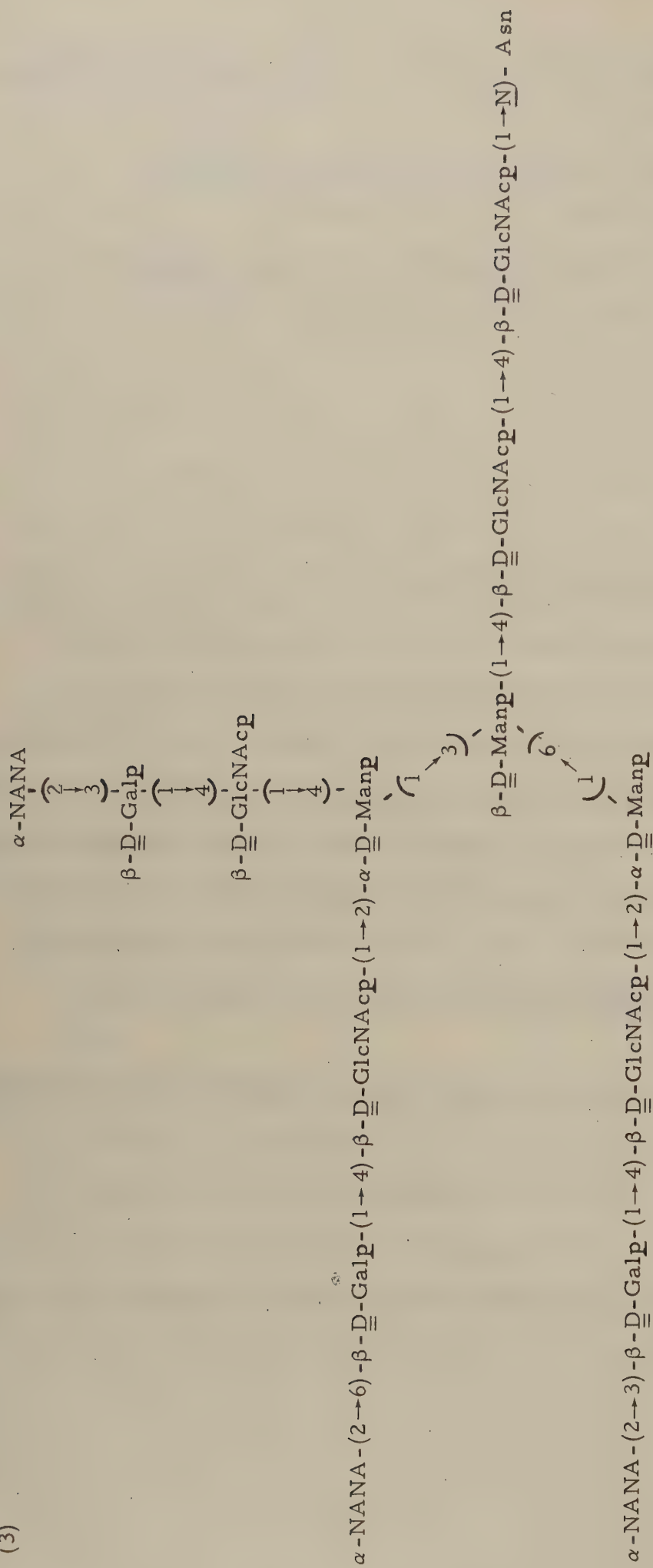
SUMMARY

The O-glycosidically linked carbohydrate chains of fetuin have been cleaved off by alkaline borohydride treatment and separated from the N-glycosidically linked carbohydrate chains still bound to the protein. Both types of carbohydrate chains have been studied by sugar and methylation analysis before and after removal of N-acetyl neuraminic acid residues. The N-glycosidically linked carbohydrate chains have been further analyzed by Smith degradation, trifluoroacetylytic degradation and degradation after chromium trioxide oxidation. As a result of these studies the following structures for the carbohydrate chains of fetuin are proposed:



and

(3)

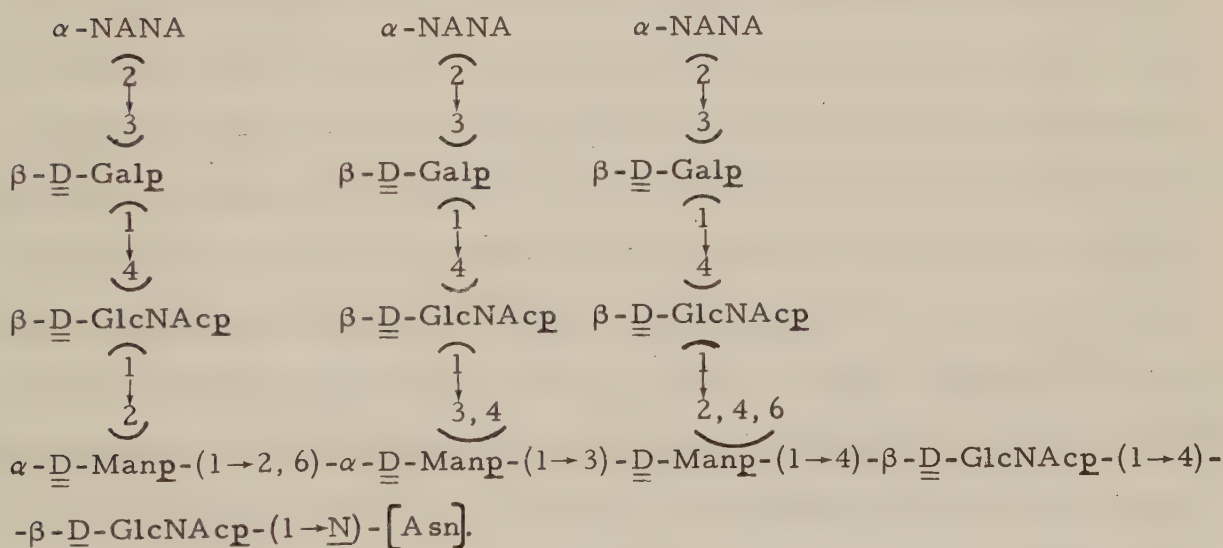


INTRODUCTION

Fetuin is the major glycoprotein in fetal calf serum. It has been shown by Spiro and co-workers (1, 2) that fetuin has three oligosaccharide chains per molecule O-glycosidically linked to serine or threonine. These carbohydrate chains have either the structure α -NANA-(2 $\rightarrow$ 3)- β -D-Galp-(1 $\rightarrow$ 3)- α -D-GalNAcp-(1 $\rightarrow$ O)-[serine or threonine]^x or α -NANA-(2 $\rightarrow$ 3)- β -D-Galp-(1 $\rightarrow$ 3)- α -D-GalNAcp-(1 $\rightarrow$ O)-[serine or threonine].

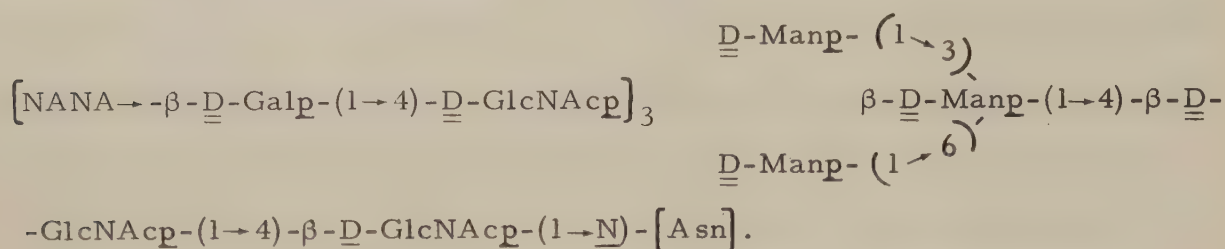


Spiro and co-workers (3) have also shown that fetuin has three carbohydrate chains per molecule N-glycosidically linked to asparagine. The structure of these carbohydrate chains has only been partially characterized. However, the following overall structure has been proposed for each chain:



^x The abbreviations used are : NANA, N-acetyl neuraminic acid; Galp, galactopyranoside; GalNAcp, 2-acetamido-2-deoxy-D-galactopyranoside; GlcNAcp, 2-acetamido-2-deoxy-D-glucopyranoside; Manp, mannopyranoside.

More recently, the following structure for the N-glycosidically linked carbohydrate chains was proposed by Bayard (4):



In the present investigation the two types of carbohydrate moieties have been studied by sugar and methylation analysis in combination with specific chemical degradation techniques.

MATERIALS AND METHODS

Analytical Methods - Colorimetric methods were used for the determination of total hexose (5), total hexosamine (6), and NANA (7). Sodium ion was determined by flame photometry. Sugar analysis was performed after hydrolysis with 90% aqueous formic acid at 100°C for 5 h followed by hydrolysis with 0.25 M aqueous sulphuric acid at 100°C for 18 h, by gas-liquid chromatography (8) and mass spectrometry (9). Methylation analysis was performed as previously described (10). Gas-liquid chromatography was carried out on a Perkin-Elmer 3920 gas chromatograph equipped with a flame ionization detector. Separations were performed on (a) an SE-30 W.C.O.T. glass capillary column (25 m x 0.25 mm) at 200°C (for partially methylated alditol acetates) and at 180°C-330°C (for permethylated oligo-saccharide alditols), (b) a glass column (2 m x 0.5 cm) packed with 3% ECNSS-M on Chromosorb Q at 200°C, 80/100 mesh (for alditol acetates). Gas-liquid chromatography-mass spectrometry was performed on a Varian MAT 311A instrument fitted with the appropriate column. The spectra were recorded at 70 eV, with an ionization current of 3 mA and an ion source temperature of 120°C. The spectra were processed on an on-line computer system (Spectrosystem 100, Varian MAT). Optical rotations were deter-

mined on a Perkin-Elmer 241 polarimeter.

Preparation of Fetuin - Fetuin was prepared according to the procedure described by Spiro (1) from fetal calf serum (Flow Labs Svenska AB, Stockholm, Sweden). The purity of the preparation was checked by polyacrylamide gel electrophoresis (11). Only minute amounts of extra components were observed. These impurities were not periodic acid Schiff positive (12) and should not therefore be glycoproteins.

Preparation of Asialofetuin - Asialofetuin was prepared from fetuin by treatment with Vibrio cholerae neuraminidase (Behringwerke AG, Marburg/Lahn, West Germany) or by mild acid hydrolysis (2). Both procedures removed more than 98% of the NANA residues.

Alkaline Borohydride Degradation of Fetuin - Alkaline borohydride degradation of fetuin and separation of the reduced oligosaccharides formed was performed as previously described by Spiro and Bhoyroo (2), except that the degradation mixture was separated into a dialysable (FD) and non-dialysable (FND) fraction before ion-exchange chromatography.

Smith Degradation of the N-Glycosidically Linked Carbohydrate Chains in Fetuin - The non-dialysable fraction (FND) obtained after alkaline borohydride treatment of fetuin and the fraction FDa (Fig. 1) were pooled and subjected to papain digestion as previously described (2). The digest was fractionated on a Sephadex G-25 (3 x 140 cm) column eluted with 0.1 M ammonium acetate buffer (pH 7.0). The void volume peak, which contained all the detectable carbohydrate material was pooled. The yield from 1 g of fetuin was 270 mg. 200 mg of this papain digested material (FNDP) was dissolved in sodium acetate buffer (0.1 M, pH 4.0) (100 ml) and to this solution was added 342 mg of sodium metaperiodate dissolved in sodium acetate buffer (0.1 M, pH 4.0) (100 ml). The periodate oxidation was carried out at 4°C in the dark and the consumption of periodate was followed at 223 nm. After 48 h no further consumption of periodate occurred so the excess was destroyed by the addition of ethylene glycol (1 ml).

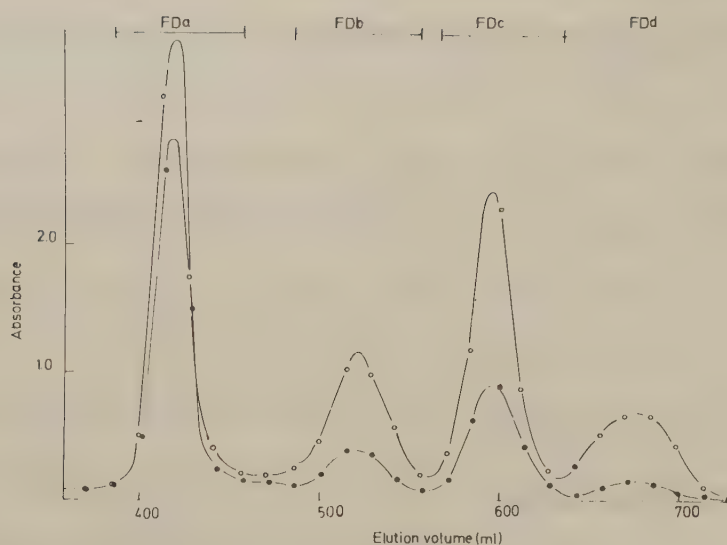


Fig. 1. Fractionation of the desalted dialysable material obtained after degradation of fetuin with alkaline borohydride on a Sephadex G-25 column (3 x 140 cm) eluted with 0.1 M ammonium acetate buffer (pH 7.0). Flow rate: 0.6 ml/min. ●—● total hexose, ○—○ sialic acid. Fractions were pooled as indicated in the figure..

Sodium borohydride (500 mg) was added in small aliquots. The reduction was carried out at 4°C for 20 h after which time the solution was acidified (pH 3) by the addition of glacial acetic acid. The solution was concentrated to dryness and boric acid was removed by codistillation with methanol (3 x 10 ml). The resulting product was desalted on a Sephadex G-25 column (3 x 140 cm) eluted with water. The yield of periodate-oxidized and reduced material was 140 mg. Part of this material was subjected to sugar and methylation analysis (FNDP IO_4^- - BH_4^-) (Tables IV and V).

Periodate-oxidized and reduced material (FNDP IO_4^- - BH_4^-) (50 mg) was hydrolyzed in 0.1 M aqueous hydrochloric acid (25 ml) for 1 h at 80°C. The hydrolysis mixture was cooled, neutralized with sodium hydrogen carbonate and reduced with sodium borodeuteride (75 mg) at 20°C for 18 h. The reaction mixture was acidified (pH 3) by the addition of glacial acetic acid and concentrated to dryness. Boric acid was removed by codistilla-

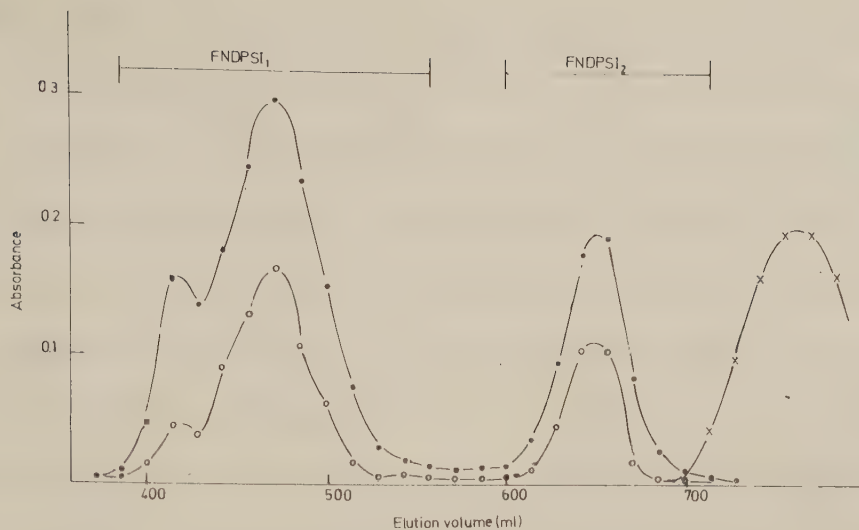


Fig. 2. Fractionation on a Sephadex G-25 column (3 x 140 cm) of the product of Smith degradation of FNDP. Eluted with water.

●—● total hexose, ○—○ hexosamine, x—x sodium ion.

tion with methanol (3 x 10 ml) and the resulting product was fractionated on a Sephadex G-25 column (Fig. 2). Peak fractions were pooled as indicated in Fig. 2. Part of fractions FNDPSI₁ was subjected to sugar and methylation analysis (Tables IV and V). The fraction FNDPSI₂ was permethylated and analyzed by gas-liquid chromatography-mass spectrometry (13) (Fig. 5). Fraction FNDPSI₁ (100 mg) was subjected to a second Smith degradation according to the procedure described above. Only the high molecular weight material was retained after fractionation on a Sephadex G-25 column (3 x 140 cm). The yield of Smith-degraded FNDPSI₁ (FNDPSII) was 70 mg. Part of FNDPSII was subjected to sugar analysis (Table IV). Fraction FNDPSII (50 mg) was dissolved in trifluoroacetic acid/trifluoroacetic anhydride (1:1, v/v, 10 ml) (sealed tube. CARE') and heated at 100°C for 48 h. The resulting dark solution was cooled and evaporated to dryness. The residue was dissolved in methanol (10 ml) and evaporated to dryness. The product was dissolved in 50% aqueous acetic acid (10 ml) and evaporated to dryness again. The residue was partitioned between diethyl ether (10 ml) and water (10 ml). The ethereal layer was

extracted twice with water (10 ml). The combined water phases were extracted once with diethyl ether (10 ml) and concentrated to 5 ml. To this solution was added sodium borodeuteride (100 mg) and the reduction was carried out at 20°C for 20 h. The reduction mixture was acidified (pH 3) with glacial acetic acid and concentrated to dryness. Boric acid was removed by codistillation with methanol (3 x 10 ml). The resulting product was acetylated in formamide (5 ml) containing pyridine (2 ml) and acetic anhydride (2 ml) for 18 h at 20°C. The acetylation mixture was diluted with ethanol (5 ml) and volatile reagents were removed by evaporation under reduced pressure. The resulting formamide solution was fractionated on a Sephadex LH-20 column (2 x 40 cm) eluted with acetone/chloroform (2:1, v/v). The acetylated carbohydrates were eluted free from formamide. After O-deacetylation the product (2.6 mg) was subjected to sugar analysis. Permethylation of the product and analysis on gas-liquid chromatography-mass spectrometry (13) showed essentially one component with the mass spectrum shown in Fig. 7.

Smith Degradation of the Desialylated N-Glycosidically Linked Carbohydrate Chains in Fetuin - FNDP was desialylated by mild acid hydrolysis as previously described for fetuin (2). The resulting asialo-FNDP (AFNDP) (200 mg) was subjected to two consecutive Smith degradations to give successively AFNDPSI₁ (Fig. 3) and AFNDPSII. Trifluoroacetylation of AFNDPSII as described above for FNDPSII gave 2.8 mg of the O-deacetylated product. The product was then subjected to sugar analysis and permethylation. The mass spectrum of the permethylated product (13) is shown in Fig. 8.

Chromium Trioxide Oxidation of the N-Glycosidically Linked Carbohydrate Chains in Fetuin - Reduced (NaBD₄) N-glycosidically bound carbohydrate chains obtained by trifluoroacetylation of asialofetuin^x (50 mg) were acetylated at 100°C in pyridine (3 ml) and acetic anhydride (3 ml) for 2 h.

^x B. Nilsson and S. Svensson, manuscript in preparation.

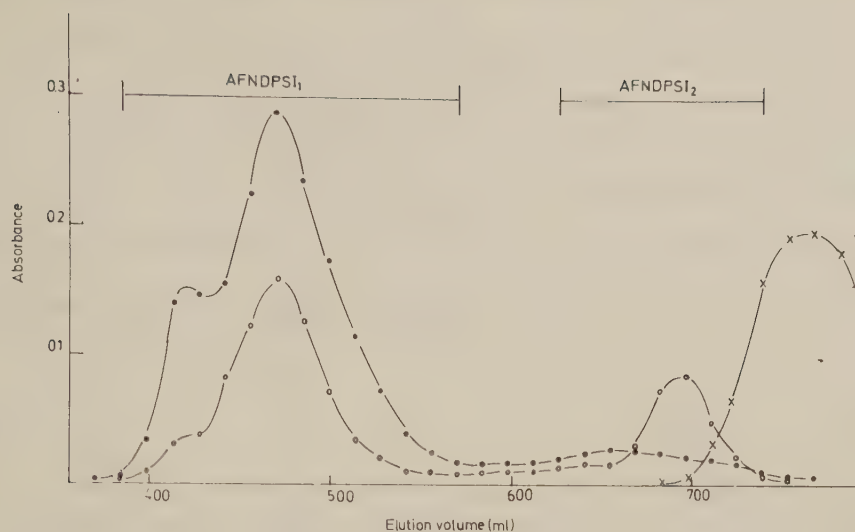


Fig. 3. Fraction on a Sephadex G-25 column of the product of Smith degradation of AFNDP. Eluted with water. ●—● total hexose, ○—○ hexosamine, x—x sodium ion.

The acetylation mixture was evaporated to dryness after addition of ethanol (2 ml) and partitioned between chloroform and water. The chloroform phase was extracted twice with water and evaporated to dryness. The yield of acetylated product was 70 mg. To about 10 mg of acetylated product was added D-xylitol pentaacetate as internal standard and the mixture was divided into two portions. One portion was subjected to sugar analysis (Table VI) and the other portion was dissolved in glacial acetic acid (10 ml). To the acetic acid solution was added chromium trioxide (100 mg) and the resulting suspension was agitated in an ultrasonic bath at 50°C for 2 h. The suspension was then cooled and diluted with water (75 ml) and the resulting solution was extracted three times with chloroform (10 ml). The combined chloroform phases were washed with water (2 x 10 ml) and evaporated to dryness. The oxidized product was then subjected to sugar analysis (Table VI). In a separate experiment 25 mg of

TABLE I

Analytical properties of fractions FDa-FDd, FND and fetuin

Fraction	Yield ^a $[\alpha]_D^{20}$	Composition %				Relative molar proportions ^b						
		NANA	D-Gal	D-Man	D-GlcNAc	D-GalNAc	NANA	D-Gal	D-Man	D-GlcNAc	D-GalNAc	
FDa	130	-	12.8	8.5	8.0	17.5	-	2.8	3.2	3.0	5.4	-
FDb	27.0	- 9.3°	56.3	16.5	-	-	16.6	2.0	1.0	-	-	0.8
FDc	32.5	-14.2°	43.7	24.5	-	-	23.6	1.0	1.0	-	-	0.8
FDD	35.6	-	+	-	-	-	-	-	-	-	-	-
FND	743	-	11.1	7.0	6.6	13.6	-	2.9	3.2	3.0	5.1	-
Fetuin	1 000	-	8.5	4.1	2.8	6.6	0.8	5.3	4.4	3.0	5.8	0.7

^a mg per g fetuin.

^b The value for D-Man is set originally to 3.0 since three D-Man residues should reside in each carbohydrate chain of fetuin.

reduced (NaBD_4) and acetylated carbohydrate chains were oxidized as described above (D-xylitol pentaacetate was omitted). The resulting product was permethylated and analyzed by gas-liquid chromatography-mass spectrometry (13). Two carbohydrate components were obtained, one monosaccharide alditol and one trisaccharide alditol. The mass spectrum of the trisaccharide alditol is shown in Fig. 9.

RESULTS

Alkaline borohydride degraded fetuin was separated into a non-dialysable (FND) and a dialysable fraction (FD). After desalting FD was further fractionated on a Sephadex G-25 column (Fig. 1). The fractions were pooled as indicated in the figure into FDa, FDb, FDC and FDD. Yields and analytical data for the fractions are given in Table I. The oligosaccharides in fraction FDb were subjected to methylation analysis before and after removal of the NANA residues. The results are summarized in Table II. The desialylated oligosaccharide in FDb and FDC gave a single peak on gas-liquid chromatography as their permethylated derivatives with identical retention times and with the same mass spectrum (13) (Fig. 4).

The non-dialysable fraction (FND) and fraction FDa were subjected to methylation analysis before and after neuraminidase treatment. Both fractions gave similar results and the data obtained for FND are summa-

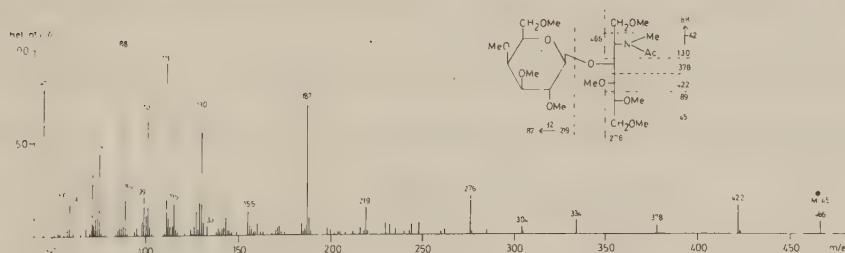


Fig. 4. Mass spectrum of the component obtained after desialylation of fractions FDb and FDC as its permethylated derivative.

TABLE II

Methyl ethers obtained in methylation analysis of oligosaccharides in fractions FDb, FDc and desialylated FDb and FDc

Sugars	T-value ^b SE-30	FDb	FDb ^d	FDc	FDc ^d
2, 3, 4, 6- <u>O</u> -Me- <u>D</u> -Gal	1.07	-	+ ^c	-	+
2, 4, 6- <u>O</u> -Me- <u>D</u> -Gal	1.55	+	-	+	-
1, 4, 5- <u>O</u> -Me- <u>D</u> -GalN(Me)Ac-OL ^a	3.70	+	-	-	-
1, 4, 5, 6- <u>O</u> -Me- <u>D</u> -GalN(Me)Ac-OL	2.10	-	+	+	+

^a 2-(N-methyl)-acetamido-2-deoxy-1, 4, 5-tri-O-methyl-D-galactitol.

^b As their corresponding alditol acetates relative to 1, 5-di-O-acetyl-2, 3, 4, 6-tetra-O-methyl-D-glucitol.

^c Quantitative determinations were not made since the response factors for the 2-(N-methyl)-acetamido-2-deoxy-D-galactitol derivatives are not known.

^d Desialylated.

rized in Table III. FND and FDa were pooled and subjected to treatment with papain in order to remove as much of the protein as possible. Sugar and methylation analysis (Tables IV and V) showed that the protease-treated material (FNDP) contained 80% carbohydrate with the same composition as FND. FND was subjected to Smith degradation and after periodate oxidation and reduction part of the material (FNDP $\text{IO}_4^- \text{-BH}_4^-$) was subjected to sugar and methylation analysis (Tables IV and V). Mild acid hydrolysis of FNDP $\text{IO}_4^- \text{-BH}_4^-$ gave a product which after reduction with sodium borodeuteride was fractionated on a Sephadex G-25 column (Fig. 2). The fractions were pooled and named as indicated in Fig. 2. The fraction

TABLE III

Methyl ethers obtained from hydrolysates of methylated FND and desialylated FND

Sugars	T-value ^a SE-30	Relative molar proportions	
		FND	FND ^b
2, 3, 4, 6- <u>O</u> -Me- <u>D</u> -Gal	1.07	0.07 (0) ^c	2.90 (3)
2, 4, 6- <u>O</u> -Me- <u>D</u> -Gal	1.55	1.77 (2)	0.05 (0)
2, 3, 4- <u>O</u> -Me- <u>D</u> -Gal	1.82	1.11 (1)	0.01 (0)
3, 4, 6- <u>O</u> -Me- <u>D</u> -Man	1.45	1.06 (1)	1.15 (1)
3, 6- <u>O</u> -Me- <u>D</u> -Man	1.97	1.19 (1)	1.10 (1)
2, 4- <u>O</u> -Me- <u>D</u> -Man	2.40	1.00 (1)	1.00 (1)
3, 6- <u>O</u> -Me- <u>D</u> -GlcN(Me)Ac	4.15	(+ (5) ^d	+ (5) ^d
3, 6- <u>O</u> -Me- <u>D</u> -GlcNAc	4.32		

^a Retention times of the corresponding alditol acetates relative to 1, 5-di-O-acetyl-2, 3, 4, 6-tetra-O-methyl-D-glucitol.

^b Desialylated.

^c Figures in parentheses represent nearest integer.

^d The relative molar proportions were calculated from the sugar analysis (Table IV).

TABLE IV

Sugar analysis of fetuin derivatives obtained after Smith degradation

Fraction	Relative molar proportions ^a			
	NANA	<u>D</u> -Gal	<u>D</u> -Man	<u>D</u> -GlcNAc
FNDP	2.9	2.9	3.0	5.1
FNDP IO ₄ ⁻ -BH ₄ ⁻	-	1.9	2.0	5.1
FNDP SI ₁	-	1.2	2.0	4.2
FNDP SII	-	-	2.0	2.9
AFNDP	-	2.9	3.0	5.2
AFNDP IO ₄ ⁻ -BH ₄ ⁻	-	0.1	2.0	5.1
AFNDP SI ₁	-	0.1	2.0	4.1
AFNDP SII	-	-	2.0	2.1

^a The value for D-Man is set originally to 3.0 since three D-Man residues should reside in each carbohydrate chain of fetuin.

TABLE V

Methyl ethers obtained in hydrolsates of permethylated fetuin derivatives after Smith degradation

Sugars	T-value ^a SE-30	Relative molar proportions ^b				
		FNDP	FNDPIO ₄ ⁻ BH ₄ ⁻	FNDPSI ₁	AFNDP	AFNDPIO ₄ ⁻ BH ₄ ⁻ AFNDPSI ₁
2, 3, 4, 6-O-Me-D-Gal	1.07	-	-	1.1	2.9	-
2, 4, 6-O-Me-D-Gal	1.55	1.8	1.8	-	0.1	-
2, 3, 4-O-Me-D-Gal	1.82	1.1	-	-	-	-
3, 4, 6-O-Me-D-Man	1.45	1.1	-	-	1.1	0.1
2, 4, 6-O-Me-D-Man	1.50	-	-	0.8	-	0.8
3, 6-O-Me-D-Man	1.97	1.0	1.0	1.0	1.0	1.0
2, 4-O-Me-D-Man	2.40	1.0	1.1	0.2	1.0	0.2
3, 4, 6-O-Me-D-GlcN(Me)Ac	3.30	-	-	+	-	+
3, 6-O-Me-D-GlcN(Me)Ac	4.15	) 5.1 ^c 5.1 ^c		+	5.2 ^c	5.1 ^c +
3, 6-O-Me-D-GlcNac	4.32					

^a Retention times of the corresponding alditol acetates relative to 1, 5-di-O-acetyl-2, 3, 4, 6-tetra-O-methyl-D-glucitol.
^b The value for 3, 6-O-Me-D-Man is set to 1.0 since one such residue should reside on each carbohydrate chain and it remains intact during the Smith degradations.
^c The relative molar proportions were calculated from the sugar analysis (Table I).

FNDPSI₂ was permethylated and analyzed by gas-liquid chromatography-mass spectrometry (Fig. 5). As can be seen from the multiple ion tracing two carbohydrate components were obtained. The mass spectra of these components are shown in Fig. 6 a and b. Fraction FNDPSI₁ was subjected to sugar and methylation analysis (Tables IV and V). Fraction FNDPSI₁ was subjected to a second Smith degradation. After desalting the high molecular weight material (FNDPSII) was subjected to sugar analysis (Table IV). FNDPSII was further degraded by trifluoroacetylation (14) and after de-O- and de-N-trifluoroacetylation (15) by reduction with sodium borodeuteride the product was acetylated and de-O-acetylated. The product was subjected to sugar analysis and permethylation. Sugar analysis gave D-Man, D-Man-OL-1-d, and D-GlcNAc in the relative molar proportions 1.0 : 1.0 : 1.1. The permethylated product was analyzed by gas-liquid chromatography-mass spectrometry (13). One major peak was obtained in the trisaccharide region with the mass spectrum shown in Fig. 7.

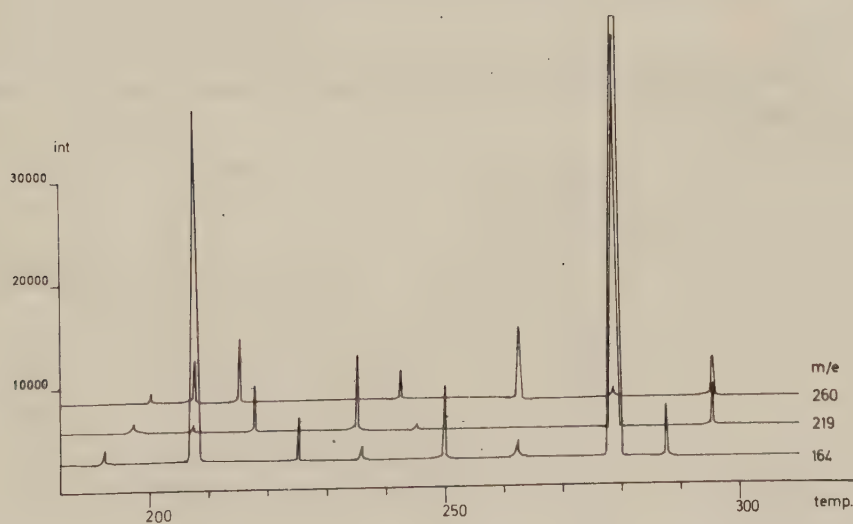


Fig. 5. Multiple ion tracing of gas-liquid chromatographic-mass spectrometric analysis of permethylated fraction FNDPSI₂. The sample was separated on an SE-30 capillary column. Temperature 180° to 330°/4 mir.

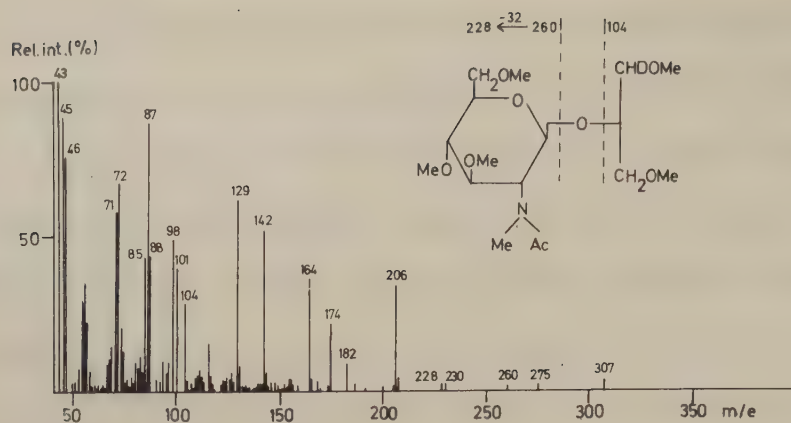


Fig. 6 a. Mass spectrum of a disaccharide alditol from permethylated fraction FNDPSI₂.

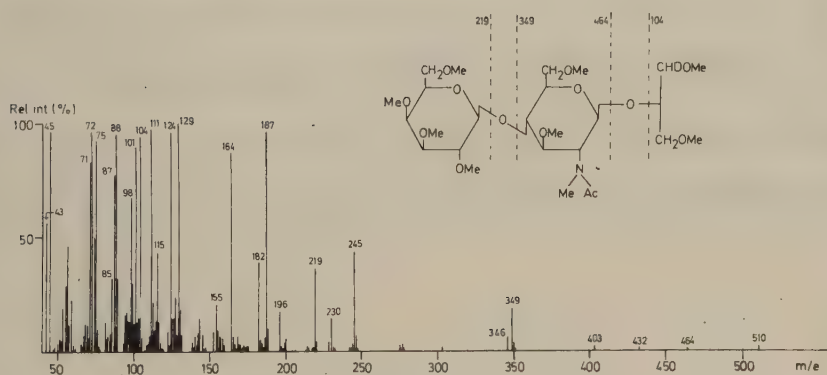


Fig. 6 b. Mass spectrum of a trisaccharide alditol from permethylated fraction FNDPSI₂.

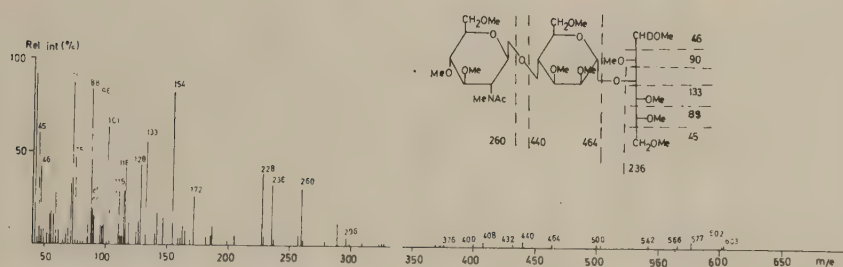


Fig. 7. Mass spectrum of the major component obtained from trifluoroacetylation of FNDPSII.

FNDP was also desialylated and subjected to Smith degradation. The product obtained after the first Smith degradation was reduced with sodium borodeuteride and fractionated on a Sephadex G-25 column (Fig. 3). Fraction AFNDPSI₂ was permethylated and analyzed by gas-liquid chromatography-mass spectrometry. Only one carbohydrate peak was obtained in the disaccharide region and had a mass spectrum identical to that in Fig. 6 a. The fraction AFNDPSI₁ was subjected to sugar and methylation analysis (Tables IV and V) and also to a second Smith degradation. The high molecular weight material (AFNDPSII) obtained after the second Smith degradation and desalting on a Sephadex G-25 column was subjected to sugar analysis (Table IV). AFNDPSII was further degraded by trifluoroacetylation (14). After de-O- and de-N-trifluoroacetylation (15) by treatment with sodium borodeuteride the product was acetylated and de-O-acetylated. Sugar analysis revealed the presence of D-Man and D-Man-OL-1-d in the relative molar proportion 1:1. The product was also permethylated and analyzed by gas-liquid chromatography-mass spectrometry (13). One major peak was obtained in the disaccharide region with the mass spectrum shown in Fig. 8.

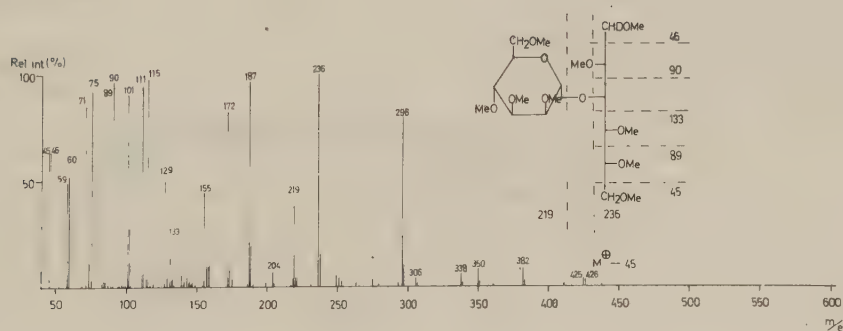


Fig. 8. Mass spectrum of a disaccharide alditol obtained after trifluoroacetylation of fraction AFNDPSII.

In order to determine the anomeric configuration of the sugar units in the N-glycosidically linked carbohydrate chains in fetuin they were released from the protein part of asialofetuin by trifluoroacetyolysis as previously described (B. Nilsson and S. Svensson, manuscript in preparation). After de-O- and de-N-trifluoroacetylation (15) by reduction with sodium borodeuteride the chains were acetylated and subjected to chromium trioxide oxidation (16). Sugar analysis of the acetylated chains was performed before and after the oxidation (Table VI).

The oxidized material was also subjected to permethylation and subsequent analysis by gas-liquid chromatography-mass spectrometry. Two main carbohydrate components were obtained of which one was identified as permethylated D-GlcNAc-OL-1-d by identical retention time on gas-liquid chromatography and mass spectrum with an authentic sample (17). The second component was a trisaccharide alditol with the mass spectrum (13) shown in Fig. 9.

TABLE VI

Sugar analysis of acetylated carbohydrate chains before and after oxidation with chromium trioxide

Sugars	Relative molar proportions	
	Before	After
<u>D</u> -Gal	3.0	0.1
<u>D</u> -Man	2.4	2.0
<u>D</u> -GlcNAc	3.6	-
<u>D</u> -Man-OL-1- <u>d</u>	0.4	0.4
<u>D</u> -GlcNAc-OL-1- <u>d</u>	0.2	0.2

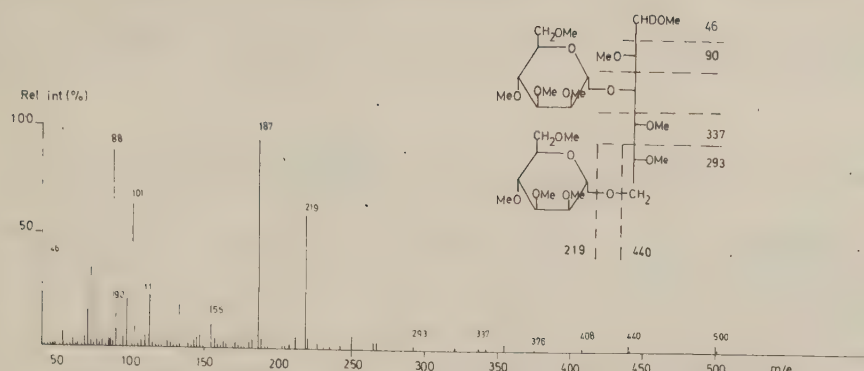


Fig. 9. Mass spectrum of the trisaccharide alditol obtained after chromium trioxide oxidation and permethylation of acetylated N-glycosidically linked carbohydrate chains of fetuin.

DISCUSSION

The O-glycosidically linked carbohydrate chains in fetuin were released from the protein by alkaline borohydride treatment. After fractionation on Sephadex G-25 the two components FDb and FDC were obtained. FDb was shown to have the structure NANA-(2→3)-D-Galp-(1→3)-[NANA-(2→6)]-D-GalNAc-OL by sugar and methylation analysis before and after desialylation (Tables I and II). Permethylation of the product obtained after desialylation of the component FDb showed a single peak on gas-liquid chromatography with the mass spectrum shown in Fig. 4. Fragments from the A-series, J-series and from cleavage of the alditol chain (Fig. 4) demonstrate that the peak represents permethylated hexp-(1→3)-hexNAc-OL (13). This finding shows homogeneity of the component FDb and corroborates the structure suggested from the sugar and methylation analysis.

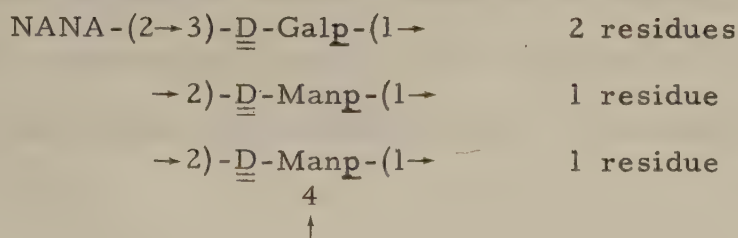
The anomeric configuration of the NANA residues should be α since they are hydrolysed by neuraminidase. The anomeric configuration of the D-Gal residue should be β since the oligosaccharide has a substantial

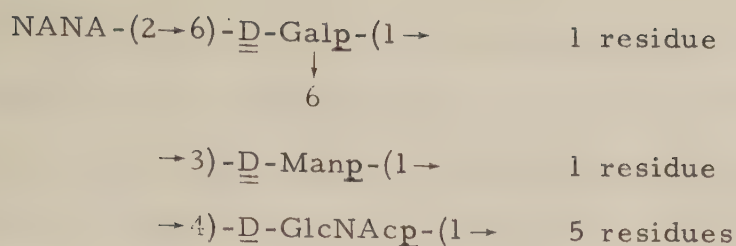
negative optical rotation (-9.3°). From the above information the structure of component FDb is α -NANA-(2 $\rightarrow$ 3)- β -D-Galp-(1 $\rightarrow$ 3)-[α -NANA-(2 $\rightarrow$ 6)]-D-GalNAc-OL.

The compound FDc has the structure NANA-(2 $\rightarrow$ 3)-D-Galp-(1 $\rightarrow$ 3)-D-GalNAc-OL as shown by sugar and methylation analysis before and after desialylation (Tables I and II). Permethylation of desialylated FDc gave a single peak on gas-liquid chromatography with a mass spectrum (13) identical to that in Fig. 4 demonstrating homogeneity of the component and further corroborating the structure. The anomeric configuration of the NANA residue should be α since it can be hydrolyzed by neuraminidase and the negative optical rotation (-14.2°) demonstrates that the D-Gal residue is β -linked. The structure for FDc is thus α -NANA-(2 $\rightarrow$ 3)- β -D-Galp-(1 $\rightarrow$ 3)-D-GalNAc-OL. Spiro and Bhoyroo (2) have previously characterized these structures and obtained a similar quantity of both. The results obtained by us thus confirm the results obtained by Spiro and Bhoyroo.

Since FDb and FDc are released by alkaline borohydride treatment at 37°C they should have been O-glycosidically linked to serine or threonine. Spiro and Bhoyroo have shown this by isolation of glycopeptides containing such carbohydrate structures (18). From the amount of D-GalNAc present in fetuin and its molecular weight it can be calculated that three O-glycosidically linked carbohydrate chains reside in each molecule of fetuin. Fraction FDd contained NANA-positive material. This fraction was not investigated further.

The N-glycosidically linked carbohydrate chains in fetuin were still bound to protein after alkaline borohydride treatment and present in fractions FND and FDa. Methylation analysis before and after desialylation showed the following structural elements in these carbohydrate chains:





In order to determine the sequential arrangement of these structural units fractions FND and FDa were pooled and deproteinized. The resulting product FNDP was subjected to Smith degradation before and after desialylation. Periodate oxidation of asialo-FNDP (AFNDP) destroyed all $\underline{\underline{\text{D}}}\text{-Gal}$ residues and one of three $\underline{\underline{\text{D}}}\text{-Man}$ units as expected (Table IV). Mild acid hydrolysis of the reduced product and reduction with sodium borodeuteride gave a product which gave two carbohydrate fractions after separation on a Sephadex G-25 column (Fig. 3). Sugar analysis of fraction AFNDPSI₁ showed that one $\underline{\underline{\text{D}}}\text{-GlcNAc}$ residue had been lost during the hydrolysis. Methylation analysis (Table V) showed that the oxidized 2-substituted $\underline{\underline{\text{D}}}\text{-Man}$ residue was cleaved off from the 6-position of the 3,6-disubstituted $\underline{\underline{\text{D}}}\text{-Man}$ unit, generating a 3-substituted $\underline{\underline{\text{D}}}\text{-Man}$ residue.

The mass spectrum of permethylated AFNDPSI₂ shown in Fig. 6a is compatible with a permethylated hexNAcp-(1→2)-glycerol-1-d (13) since it shows, although weak, the A-series of fragments, $\underline{m/e}$ 260 and $\underline{m/e}$ 228, and a J-series of peaks at $\underline{m/e}$ 104 and $\underline{m/e}$ 164. The fragment at $\underline{m/e}$ 307 represents loss of ketene and a methoxy radical from the molecular ion ($\underline{m/e}$ 380) which is not visible. The ion at $\underline{m/e}$ 307 can then lose a molecule of methanol forming the ion at $\underline{m/e}$ 275. The fragment at $\underline{m/e}$ 206 can be formed from the E₁ fragment by the transformation shown in Fig. 10 and by a further loss of methanol the ion $\underline{m/e}$ 174 is formed. The fragments at $\underline{m/e}$ 142 and $\underline{m/e}$ 129 belong to the F- and H-series of ions. The (1→2)-linkage is supported by the presence of an ion at $\underline{m/e}$ 46 and absence of fragments at $\underline{m/e}$ 89, $\underline{m/e}$ 59 and $\underline{m/e}$ 90, $\underline{m/e}$ 60, $\underline{m/e}$ 59 that should have been formed if the linkage had been (1→1) or (1→3), respectively. Since the component was produced by cleavage of the (1→2)-substituted $\underline{\underline{\text{D}}}\text{-Man}$ residue

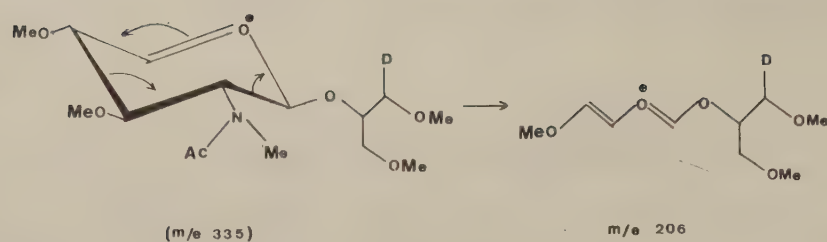


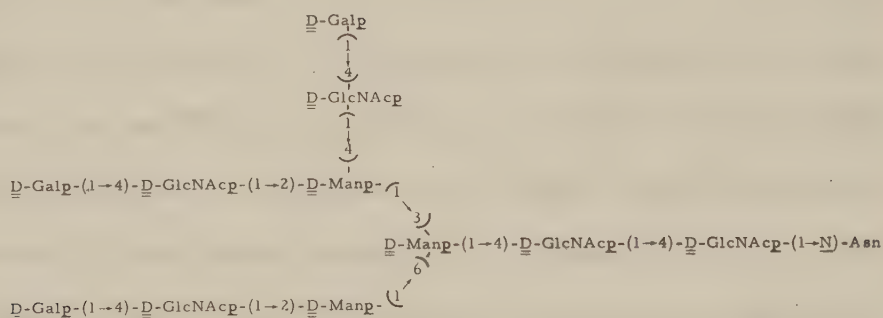
Fig. 10. Mechanism for formation of the fragment m/e 206 in Fig. 6 a.

and since D-GlcNAc is the only hexNAc present, the structure of the di-saccharide component in fraction AFNDPSI₂ is D-GlcNAc_p-(1→2)-glycerol-1-d. From the information obtained in one Smith degradation of AFNDP the sequence D-Gal_p-(1→4)-D-GlcNAc_p-(1→2)-D-Man_p-(1→ has been demonstrated and further that this unit or a D-Gal_p-(1→ residue is linked to the D-Man_p-(1→ residue. When AFNDPSI₁ was subjected to periodate oxidation two of the D-GlcNAc residues were destroyed, as expected (Table IV). After mild acid hydrolysis a high molecular component (AFNDPSII) was obtained which had D-GlcNAc and D-Man residues in the relative molar proportions 2:2. Due to the low molecular weight of the material the methylated product could not be purified by dialysis and therefore methylation analysis was not attempted. The material AFNDPSII was instead degraded by trifluoroacetylation (14) at conditions which will degrade the protein, cleave the N-glycosidic bond and degrade any reducing D-GlcNAc residue formed. The material obtained after trifluoroacetylation was treated with acetic acid and reduced with sodium borodeuteride to effect de-O- and de-N-trifluoroacetylation (15). The reduction step will also convert any reducing sugar residue to its corresponding alditol-1-d. The product obtained was acetylated and de-O-acetylated. Sugar analysis showed only D-Man and D-Man-OL-1-d in roughly equal amounts. After

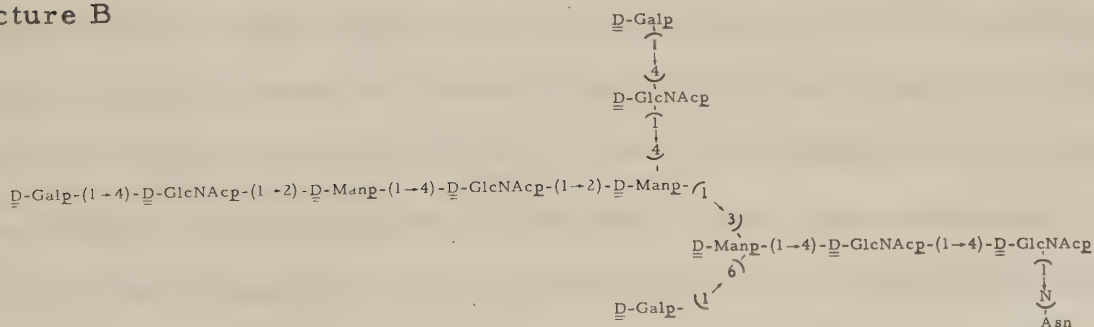
permethylation gas-liquid chromatographic-mass spectrometric analysis showed one major component in the disaccharide region and only traces of trisaccharides. The mass spectrum of the permethylated disaccharide alditol was typical for $\text{hexp-(1}\rightarrow\text{3)-hexitol-1-d}$ (13) (Fig. 8). From this information, the product obtained after two consecutive Smith degradations and trifluoracetolysis followed by reduction and N-acetylation consisted mainly of $\underline{\underline{\text{D}}}\text{-Manp-(1}\rightarrow\text{3)-}\underline{\underline{\text{D}}}\text{-Man-OL-1-d}$. The structure of the carbohydrate portion in AFNDPSII must then mainly be $\underline{\underline{\text{D}}}\text{-Manp-(1}\rightarrow\text{3)-}\underline{\underline{\text{D}}}\text{-Manp-(1}\rightarrow\text{4)-}\underline{\underline{\text{D}}}\text{-GlcNAcp-(1}\rightarrow\text{4)-}\underline{\underline{\text{D}}}\text{-GlcNAcp-(1}\rightarrow\text{N)-Asn}$.

The trifluoracetolysis degradation will cleave the GlcNAc-(1→N)-Asn bond and by an eliminative degradation process cleave off the two D-GlcNAc residues starting from the reducing end (14). We have previously shown that glycosidic bonds (19) and reducing sugars with a free hydroxyl in the 2-position (14) are stable under trifluoracetolysis conditions used here. From the information obtained by Smith degradation and trifluoracetolysis degradation three possible structures for the N-glycosidically linked carbohydrate chains in asialo fetuin, can be constructed:

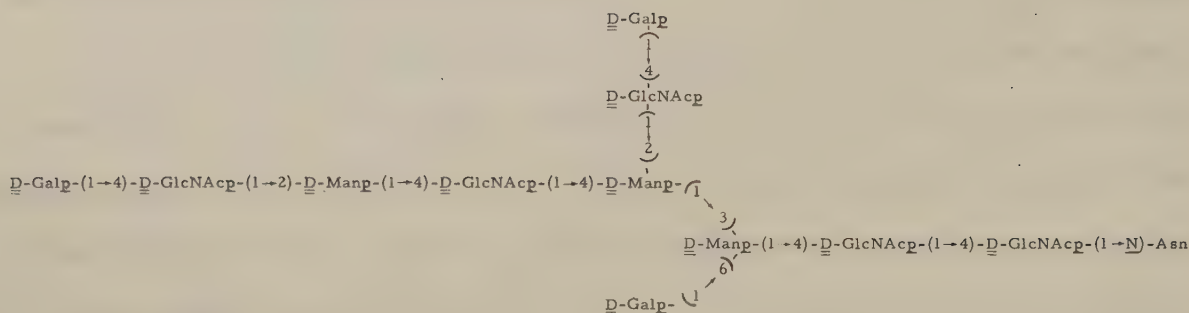
Structure A



Structure B



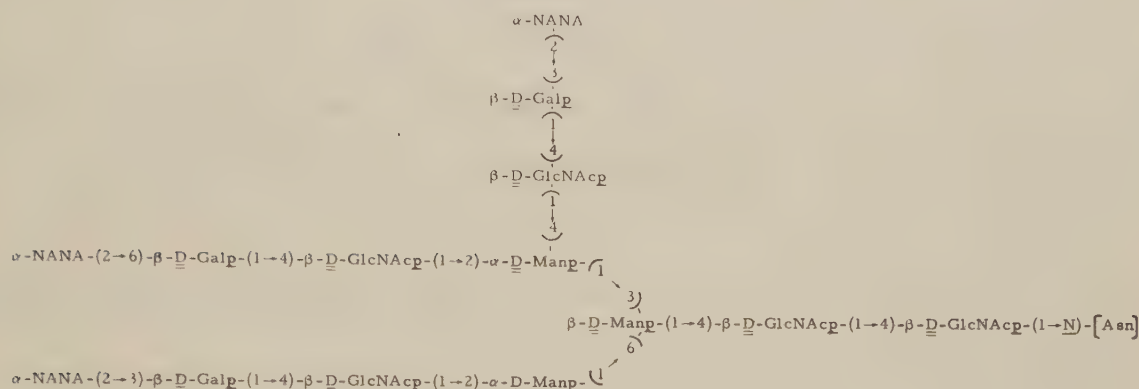
Structure C



In order to determine the anomeric configuration for the sugar residues of the N-glycosidically-linked carbohydrate chains, asialofetuin was degraded by trifluoroacetylolysis and the N-glycosidically-linked carbohydrate chains were isolated as previously described (B. Nilsson and S. Svensson, manuscript in preparation). The carbohydrate chains obtained this way had their reducing ends partly degraded so that 40% ended with a 3,6-disubstituted D-Man residue as D-Man-OL-1-d, 40% with a 4-substituted D-GlcNAc-OL-1-d residue directly bound to the 3,6-substituted D-Man unit, and the remaining with a 4-substituted D-GlcNAc-OL-1-d residue bound to the previous D-GlcNAc unit. The carbohydrate chains obtained this way consisted of more than 95% carbohydrate. The material was acetylated and subjected to chromium trioxide oxidation (16) which will oxidize acetylated sugar residues with the aglycone equatorial into 5-hexosulonsate units, whereas the sugar residues with the aglycone axial and acetylated alditols are stable. Almost all D-Gal and D-GlcNAc units and 25% of the D-Man residues were oxidized demonstrating that the D-Gal, D-GlcNAc and 25% of the D-Man residues are β -linked. Methylation of the oxidized material cleaves all ester linkages and permethylates the products formed. Direct analysis by gas-liquid chromatography-mass spectrometry of the permethylated product revealed two components. One component gave a mass spectrum typical for 1,3,4,5,6-penta-O-methyl-

component. The two peaks were obtained and the mass spectrum of the component in the trisaccharide region (Fig. 6 b) is compatible with the expected structure. The two components were obtained in the ratio 1:10. Fraction FNDPSI₁ had lost about 1.9 D-Gal units, of which 1.1 residues were destroyed by the periodate oxidation and about 1.0 D-GlcNAc residue of which none was destroyed by the periodate oxidation. Further the 2-substituted D-Man residue was oxidized and after the reduction and mild acid hydrolysis the residue was cleaved off transforming the 3,6-substituted D-Man residue into a 3-substituted chain unit. This Smith degradation demonstrates that the NANA unit linked to the chain cleaved off is mainly 3-linked to the terminal D-Gal residue. Periodate oxidation of FNDPSI₁ destroyed the residual D-Gal units and one D-GlcNAc residue (Table IV). After reduction and mild acid hydrolysis the product FNDPSII contained D-GlcNAc and D-Man in the relative molar proportions 3.1:2.0. This material was subjected to trifluoroacetylolysis as described above. Sugar analysis after reduction and N-acetylation gave the ratio D-Man:D-GlcNAc = 2.0:1.1. Permethylation of the product showed one major peak in the trisaccharide region when analyzed by gas-liquid chromatography-mass spectrometry. Its mass spectrum (Fig. 7) was typical for a permethylated hexNAcp-hexp-hexitol-1-d from the A-series and J-series of fragments (13) (Fig. 7). The linkage to the hexitol residue was determined by inspection of the fragments obtained by cleavage of the alditol chain and was found to be (1→3). The linkage between the hexNAcp and hexp residue is more difficult to ascertain but if the component is pure it should be (1→4) since elimination of methanol from abaldJ₂ (m/e 440) giving (abaldJ₂-32) (m/e 408) and abaldJ₂-2x32 (m/e 376) gives these fragments in the ratio of 5:4:1 (J. Lundsten and S. Svensson, manuscript in preparation) which is typical for a abaldJ₂ fragment with the positive charge in the 4-position. If the linkage had been (1→2) the intensity ratios between these fragments normally would be 1:8:1.

Thus the main product obtained after two Smith degradations of FNDP followed by trifluoracetolysis, de-O- and de-N-trifluoracetylation (15) by reduction with NaBD₄ and re-N-acetylation is D-GlcNAc_p-(1→4)-D-Man_p-(1→3)-D-Man-OL-1-d. From the above data the structure of the N-glycosidically linked carbohydrate chains of fetuin is proposed to be:



It is of considerable biological interest to have a detailed knowledge of the structure of the carbohydrate portion of glycoproteins and other glycoconjugates, since the carbohydrate chains in these molecules are most probably involved in recognition phenomena in the organism (20). Most asialoglycoproteins investigated including asialofetuin are recognized by a liver receptor for asialoglycoproteins (20). However asialotransferrin is a remarkable exception. It has a very low affinity to the receptor in spite of the fact that it fulfils the known structural requirements for binding (21).

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A NEW METHOD FOR DEGRADATION OF THE PROTEIN PART OF GLYCOPROTEINS. ISOLATION OF THE CARBOHYDRATE CHAINS OF ASIALOFETUIN

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ABSTRACT

A new method has been developed with which the protein part of several glycoproteins can be degraded while leaving the carbohydrate portion virtually intact but partially degraded at the reducing end. The method is based upon stabilization of the glycosidic linkages of the sugar moieties by trifluoracetyl groups and subsequent cleavage of the peptide bonds by transamidation. The two reactions are carried out in a mixture of trifluoroacetic anhydride and trifluoroacetic acid. After de-O- and de-N-trifluoroacetylation the carbohydrate portion can be isolated and the original N-acetyl functions reconstituted. The applicability of the method is demonstrated by the isolation of the N- and O-glycosidically linked carbohydrate chains of asialofetuin.

INTRODUCTION

The carbohydrate chains of glycoproteins are linked to the protein part either N-glycosidically to asparagine or O-glycosidically to serine, threonine, hydroxylysine or hydroxyproline¹. The carbohydrate chains can be isolated as glycopeptides by extensive action of proteolytic enzymes

but often the peptide portion left is of considerable size. N-Glycosidically linked carbohydrate chains can be cleaved off by hydrazinolysis² or by alkaline borohydride treatment³ and O-glycosidically linked carbohydrate chains, linked to serine or threonine, by mild alkaline borohydride treatment⁴.

In the present communication we wish to report a new method to degrade the protein part of glycoproteins leaving the carbohydrate portion virtually unaffected, but partially degraded at the reducing end.

RESULTS

When asialofetuin was treated with trifluoroacetic anhydride/trifluoroacetic acid (TFAA/TFA) in the proportions 50:1 at 100°C it dissolved within 3 h giving a dark, clear solution. After 48 h the reaction was

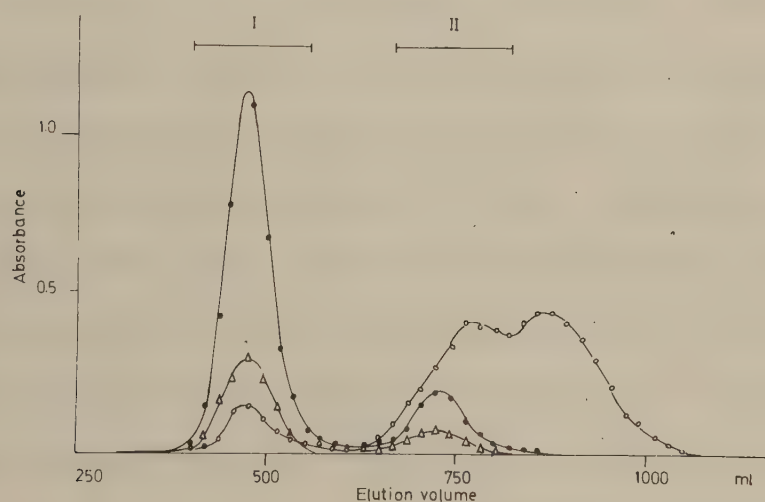


Fig. 1. Fractionation of asialofetuin on a Sephadex G-25 column (3 x 140 cm) after reaction with TFAA/TFA and de-O-trifluoroacetylation. Eluant: 0.1 M ammonium acetate buffer, pH 7.0. The flow rate was 0.5 ml/min and fractions (7.6 ml) were assayed for total hexose ●—●, hexosamine △—△ and material absorbing at 280 nm ○—○. Fractions were pooled as indicated in the figure.

stopped and the reagents were removed by evaporation leaving a residue consisting of O- and N-trifluoracetylated carbohydrates and degradation products. After de-O-trifluoracetylation by treatment with methanol and 50% aqueous acetic acid the N-trifluoracetylated product was freed from degradation products by extraction with diethyl ether. The resulting product was then fractionated on a Sephadex G-25 column (Fig. 1).

The two fractions were then de-N-trifluoracetylated by reduction with sodium borodeuteride, acetylated and de-O-acetylated. Each fraction was then purified by gel filtration (Figs. 2 and 3).

Yields and analytical data for the fractions IR and IIR, obtained after pooling (see Figs. 2 and 3) are given in Table I. Fraction IR was further characterized by methylation analysis (Table II). Fraction IIR gave after acetylation 4% of D-galactitol-1-d hexaacetate. Fraction IIR was also permethylated and analysis by g.l.c.-m.s. revealed a single component in the disaccharide region with the mass spectrum shown in

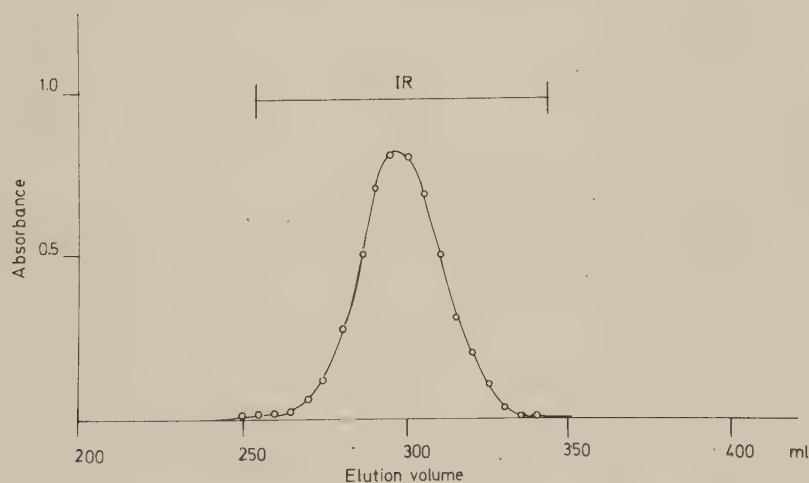


Fig. 2. Fractionation of fraction I after reduction with sodium borodeuteride and N-acetylation on a Sephadex G-50 column (2.5 x 80 cm), eluted with water. The flow rate was 0.5 ml/min and fractions (5.0 ml) were assayed for total hexose.

TABLE I

YIELDS AND ANALYTICAL DATA FOR RECONSTITUTED FRACTIONS OBTAINED FROM TRIFLUORACETOLYSIS OF ASIALOFETUIN

Fraction	Yield (mg)	Composition (%)			
		<u>D</u> -Gal	<u>D</u> -Man	<u>D</u> -GlcNAc	<u>D</u> -GalNAc-OL-1- <u>d</u> <u>D</u> -GalNAc
IR	150.3	26.5 (3.2) ^a	24.8 ^b (3.0)	41.5 ^c (4.1)	-
IIR	50.0	22.0 ^d (1.4)	-	-	18.5 (1.0)
Asialofetuin	1 000	4.5 (4.1)	3.3 (3.0)	7.1 (5.3)	1.1 (0.8)

^a Molar ratios in brackets.

^b This value also includes D-Man-OL-1-d.

^c This value also includes D-GlcNAc-OL-1-d.

^d 4 of this 22% represent free D-Gal-OL-1-d.

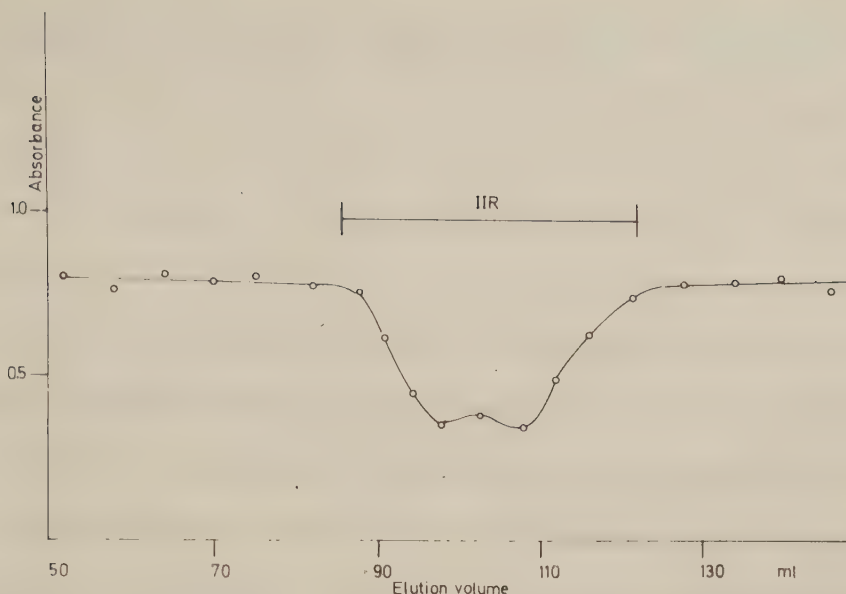


Fig. 3. Fractionation of fraction II after reduction with sodium borodeuteride and N-acetylation on a Sephadex G-25 column (2 x 45 cm), eluted with water. The flow rate was 0.3 ml/min and fractions (3.0 ml) were assayed for consumption of periodate at 223 nm.

TABLE II

METHYL ETHERS OBTAINED IN HYDROLYSATE OF REDUCED N-ACETYLATED AND METHYLATED FRACTION IR AND ASIALOFETUIN

Sugars	T-value SE-30 ^a	Molar ratios	
		Fraction IR	Asialo- fetuin
2, 3, 4, 6- <u>O</u> -Me- <u>D</u> -Gal	1.07	2.9	3.0
3, 4, 6- <u>O</u> -Me- <u>D</u> -Man	1.45	1.3	1.1
3, 6- <u>O</u> -Me- <u>D</u> -Man	1.97	1.0	1.0
2, 4- <u>O</u> -Me- <u>D</u> -Man	2.40	0.6	1.0
3, 6- <u>O</u> -Me- <u>D</u> -GlcN(Me)Ac	4.15	) +	) 5.3
3, 6- <u>O</u> -Me- <u>D</u> -GlcNAc	4.32		
1, 2, 4, 5- <u>O</u> -Me- <u>D</u> -Man-OL-1-d	1.00	0.4	-
1, 3, 5, 6- <u>O</u> -Me- <u>D</u> -GlcN(Me)Ac-OL-1-d	1.95	) +	) -
1, 3, 5, 6- <u>O</u> -Me- <u>D</u> -GlcNAc-OL-1-d	2.95		

^a Retention times of the corresponding alditol acetates relative to 1, 5-di-O-acetyl-2, 3, 4, 6-tetra-O-methyl-D-glucitol.

Fig. 4. This mass spectrum shows fragments typical for a permethylated hexp-(1→3)-hexNAc-OL-1-d⁵ (Fig. 5).

DISCUSSION

We have shown⁶ that O-glycosidically linked sugars are stable to the action of TFAA/TFA 50:1 at 100°C for 48 h. This stability of the glycosidic bond is due to the inductive effect of the O-trifluoracetyl groups of the pertrifluoracetylated sugars rapidly formed in the reagent mixture. The greatest stabilizing effect is given by the 2-O-trifluoracetyl group in the

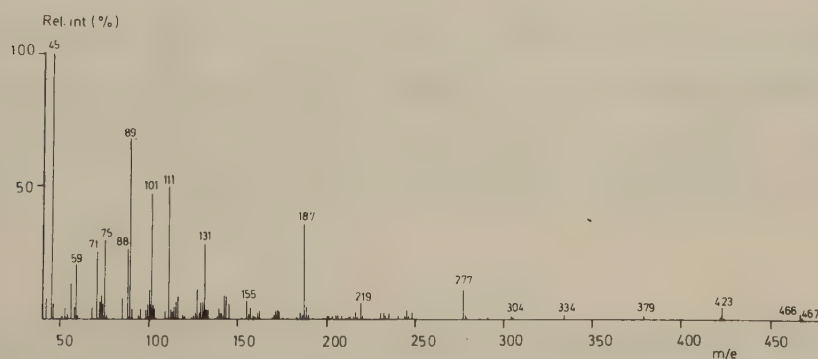


Fig. 4. Mass spectrum of the permethylated disaccharide alditol obtained from permethylation of fraction IIR.

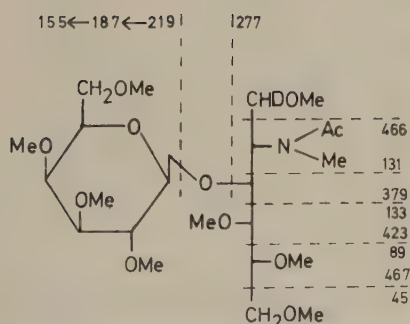
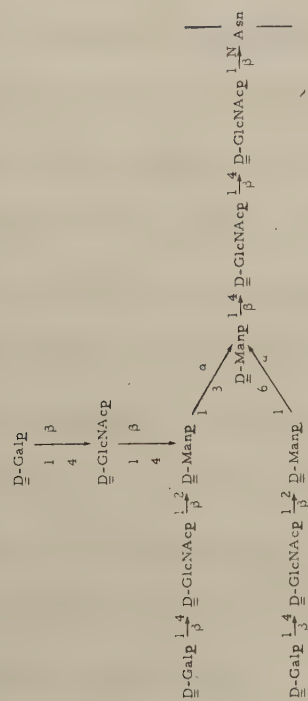


Fig. 5. Characteristic fragmentation for a permethylated hexp-(1→3)-hexNAc-OL-1-d.

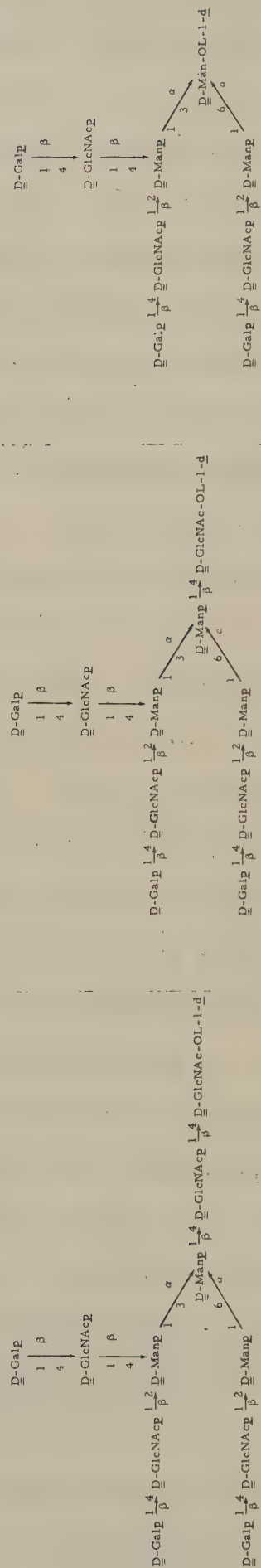
glycone and the O-trifluoracetyl groups adjacent to the glycosidic linkage in the aglycone sugar residue, in analogy with results obtained from aceto-lysis experiments⁷. The reagent TFAA/TFA, however, transamidates the N-acetyl function of 2-acetamido-2-deoxy sugars⁸, and thus also peptide linkages, to form N-trifluoracetyl derivatives within 48 h at 100°C.

We have now investigated the possibility of degrading the protein part of glycoproteins and recovering the carbohydrate chains intact and free from protein. Since the structures of the carbohydrate chains of fetuin have been extensively studied⁹⁻¹², asialofetuin was chosen as a model. Asialofetuin was degraded with TFAA/TFA 50:1 to yield an O- and N-trifluoracetylated product. After de-O-trifluoracetylation the N-trifluoracetylated carbohydrates were recovered and fractionated by gel filtration (Fig. 1). Since we wanted to reconstitute the original N-acetyl groups of the 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-galactose residues, the N-trifluoracetyl groups were removed by reduction with sodium borodeuteride⁸. The reduction step also converts a reducing sugar unit into an alditol residue labelled with a deuterium atom at C-1. N-Acetylation was carried out in two steps, i.e. O- and N-acetylation followed by de-O-acetylation.

Degradation with TFAA/TFA 50:1 resulted in degradation of the protein and elimination of the N-glycosidically linked carbohydrate chains by cleavage of the 2-acetamido-1-N-(4'-L-aspartyl)-2-deoxy-D-glucopyranosylaminic bond. The initially formed pertrifluoracetylated 4-O-substituted 1-N- β -trifluoracetyl-2-deoxy-2-trifluoracetamido-D-glucopyranosylamine residue at the reducing end is then further partially degraded by acid catalyzed elimination generating a pertrifluoracetylated 4-O-substituted 2-deoxy-2-trifluoracetamido-D-glucose residue at the reducing end. This residue is in its turn partly degraded¹³ exposing the 3,6-di-O-substituted D-mannose residue which is stable (Scheme 1). The product was de-O-trifluoracetylated by treatment with methanol and 50% aqueous acetic acid



1. TFAA/TFA
2. 50% acetic acid
3. NaBD_4
4. N-acetylation

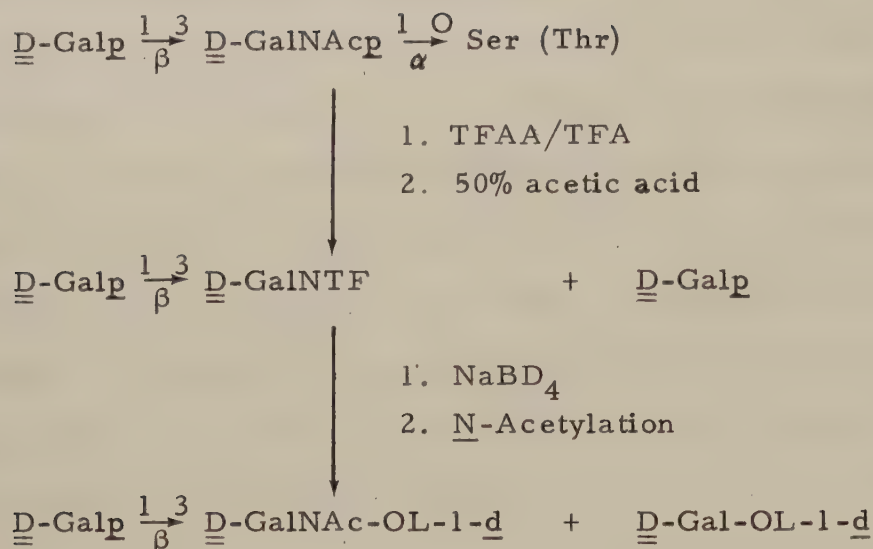


and de-N-trifluoracetylated by reduction with sodium borodeuteride. The reduction step also converts the 3,6-disubstituted D-mannose residue and the 4-O-substituted 2-amino-2-deoxy-D-glucose unit into their corresponding alditols-1-d (Table II). The remaining 4-O-substituted 1-N- β -trifluoracetyl-2-deoxy-2-trifluoracetamido-D-glucopyranosylamine residues should be converted into 4-O-substituted 2-amino-2-deoxy-D-glucitol-1-d residues in analogy with model experiments with TFAA/TFA 50:1 and reduction with sodium borodeuteride on D-glucopyranosylamine pentaacetate¹⁴. Methylation analysis (Table II) and gel filtration (Fig. 2) shows that the N-glycosidically linked carbohydrate chains are intact with the modifications of the reducing end discussed above.

TFAA/TFA also cleaved off the O-glycosidically linked carbohydrate chains yielding fraction II (Fig. 1). Removal of the N-trifluoracetyl groups and re-N-acetylation of II yielded a product which gave two partly resolved peaks on G-25 (Fig. 3). Sugar analysis of IIR (Fig. 3) (Table I) and analysis of the permethylated IIR showed that it consisted of the components D-Gal-OL-1-d (4%) and D-Galp $\xrightarrow[1]{3}\beta$ D-GalNAc-OL-1-d (40%). No attempt was made to further purify this fraction. The cleavage of the linkage between the 3-O-substituted 2-acetamido-2-deoxy-D-galactose residue and serine (or threonine) probably involves acid catalyzed elimination¹⁵ (Scheme 2) and trifluoracetylation of the C-1 hydroxyl formed. The reducing 2-acetamido-2-deoxy-D-galactose residue is further partially degraded¹³ generating pertrifluoracetylated D-galactose. The degradation and reconstitution of the O-glycosidically linked carbohydrate chains in asialofetuin are shown in Scheme 2.

The present study shows that the carbohydrate chains of asialoglycoproteins can be cleaved off by TFAA/TFA 50:1. The N-glycosidically linked carbohydrate chains are cleaved off by transamidation of the D-GlcNAc $\xrightarrow[1]{N}\beta$ Asn linkage and the O-glycosidically linked carbohydrate chains are cleaved off probably by acid catalyzed elimination from the

Scheme 2



serine (or threonine) residues. The carbohydrate chains obtained have been partly degraded at the reducing end. Since the protein part is degraded by transamidation into low molecular products the carbohydrate chains can be isolated in a reasonable pure state by gel filtration.

MATERIALS AND METHODS

General methods. - Evaporation was performed under reduced pressure at bath temperatures not exceeding 40°C. G.l.c. was carried out on a Perkin-Elmer 3920 gas chromatograph equipped with a flame ionization detector. Separations were performed on (a) OV 101 W.C.O.T. glass capillary columns (25 m x 0.25 mm) at 200°C (for partially methylated alditol acetates), (b) SE-30 W.C.O.T. glass capillary columns (25 m x 0.25 mm) at 230°C (for partially methylated 2-acetamido-2-deoxy-alditol acetates) or 180-330°C (for permethylated oligosaccharide alditols) and (c) glass columns (2 m x 0.5 cm) packed with 3% ECNSS-M on Chromosorb Q at 200°C (for alditol acetates). G.l.c.-m.s. was performed on a Varian MAT 311A instrument fitted with the appropriate column. The

spectra were recorded at 70 eV, with an ionization current of 3 mA and an ion source temperature of 120°C. The spectra were processed by an on-line computer system (Spectrosystem 100, Varian MAT).

Analytical methods. - Colorimetric methods were used for the determination of total hexose¹⁶, total hexosamine¹⁷ and sialic acid¹⁸. Sugar analysis was performed by g.l.c.¹⁹ and m.s.²⁰ after hydrolysis with 90% aqueous formic acid at 100°C for 5 h followed by hydrolysis with 0.25 M aqueous sulphuric acid at 100°C for 18 h. Methylation analysis was performed as previously described²¹.

Asialofetuin. - Asialofetuin was prepared from fetuin by mild acid hydrolysis as previously described⁹. The preparation contained less than 1% of sialic acid¹⁸.

Degradation of asialofetuin. - A solution of TFAA/TFA (50 ml, 50:1, v/v) was added to freeze-dried asialofetuin (1.0 g) in a sealed glass vessel (250 ml). The reaction mixture was heated at 100°C for 48 h (Caution: corrosive mixture under pressure). The resulting clear, dark solution was cooled and evaporated to dryness. The product was dissolved in methanol (25 ml) and evaporated to dryness again. The product was partitioned between diethyl ether (25 ml) and water (25 ml). The ethereal layer was extracted twice with water (25 ml). The combined water phases were extracted once with diethyl ether (25 ml) and evaporated to 10 ml (n-octanol was added to prevent foaming). The water solution was fractionated on a Sephadex G-25 (fine) column (3 x 140 cm) eluted with 0.1 M ammonium acetate buffer pH 7.0. The separation was monitored by analysis of total hexose, hexosamine and ultraviolet absorption at 280 nm (Fig. 1). Fraction I (see Fig. 1) (200 mg) was dissolved in water (20 ml) and reduced for 5 h, at room temperature, with sodium borodeuteride (100 mg). The reaction mixture was acidified with glacial acetic acid and evaporated to dryness. Boric acid was removed by three codistillations with methanol (10 ml) and the resulting product was desalted on a

Sephadex G-25 column (3 x 140 cm) eluted with distilled water. The product obtained (170 mg) was dissolved in formamide (10 ml) and a mixture of pyridine and acetic anhydride (10 ml, 1:1, v/v) was added. After 20 h at room temperature ethanol (10 ml) was added to the acetylation mixture. Volatile reagents were then removed by evaporation at reduced pressure. The resulting formamide solution was fractionated on a Sephadex LH-20 column (3 x 50 cm) eluted with acetone:chloroform (2:1, v/v) (flow rate: 5 ml/min). The acetylated carbohydrates were eluted with the void volume, free from formamide. The yield of acetylated product was 250 mg. The acetylated product was de-O-acetylated by 1 M ammonia in 75% aqueous methanol (50 ml) at room temperature for 18 h. The reaction mixture was evaporated to dryness and the residue was fractionated on a Sephadex G-50 column (2.5 x 80 cm) eluted with distilled water (Fig. 2). Fractions were pooled as indicated in Fig. 2 and lyophilized to yield reduced and N-acetylated fraction I.

Fraction II (see Fig. 1) was reduced with sodium borodeuteride (50 mg) in water (10 ml) for 18 h. The reduction mixture was acidified with glacial acetic acid and evaporated to dryness. Boric acid was removed by codistillations with methanol (3x 10 ml). The reduced fraction II was acetylated with pyridine (5 ml) and acetic anhydride (5 ml) at 100°C for 30 min. Excess acetic anhydride was destroyed by the addition of ethanol (5 ml) and the resulting solution was evaporated to dryness. The residue was partitioned between water (25 ml) and chloroform (25 ml), the chloroform layer was washed with water (3 x 25 ml) and evaporated to dryness. The acetylated product was de-O-acetylated by 1 M ammonia in 75% aqueous ethanol (10 ml), at room temperature, for 18 h and evaporated to dryness. The residue was fractionated on a Sephadex G-25 column (2 x 45 cm) (Fig. 3). Fractions were pooled as indicated in Fig. 3 to yield reduced and N-acetylated fraction II.

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